PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 15/00, 7/00 // A61K 39/04 G01N 33/68 — A1

(11) International Publication Number: WO 88/ 02027

(43) International Publication Date: 24 March 1988 (24.03.88)

(21) International Application Number: PCT/US87/02245

(22) International Filing Date: 4 September 1987 (04.09.87)

(31) Priority Application Number: 904,661

(32) Priority Date: 8 September 1986 (08.09.86)

(33) Priority Country: US

(71) Applicant: APPLIED BIOTECHNOLOGY, INC. [US/US]; 80 Rogers Street, Cambridge, MA 02142 (US).

(72) Inventors: PANICALI, Dennis, L. ; 114 Nonset Path, Acton, MA 01720 (US). SKARNES, William, C. ; 275 Dutton Road, Sudbury, MA 01776 (US). MAHR, Anna, M. ; 22 Farm Hill Road, Natick, MA 01760 (US).

(74) Agents: DeCONTI, Giulio, A., Jr. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

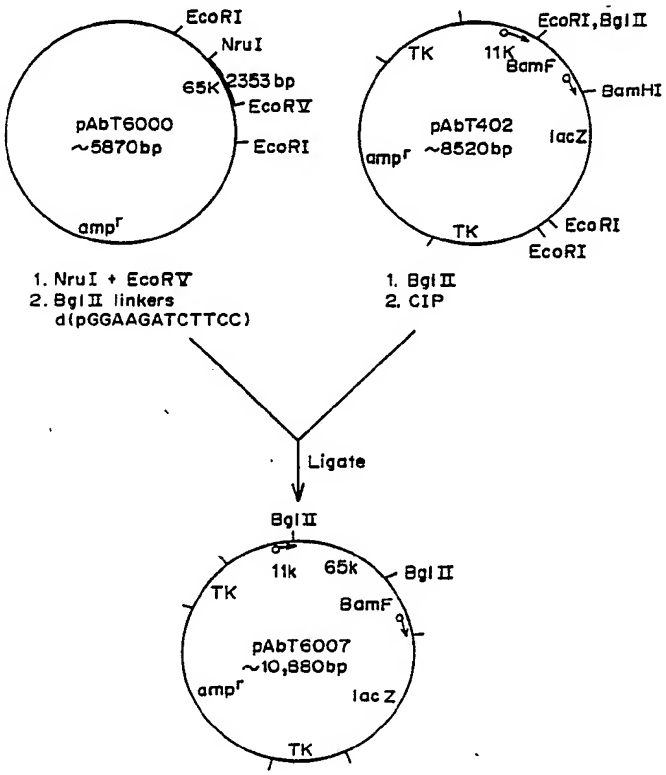
(81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), MW, NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETICALLY ENGINEERED VACCINES FOR MYCOBACTERIA

(57) Abstract

Recombinant pox viruses capable of expressing mycobacterial antigens and eliciting protective immune response against mycobacteria. The viruses are useful as live vaccines against mycobacteria.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

GENETICALLY ENGINEERED VACCINES FOR MYCOBACTERIA

Background

The mycobacteria are a group of organisms which range from innocuous and ubiquitious inhabitants of water and soil to the highly pathogenic organisms which are responsible for tuberculosis and leprosy. These are both chronic infectious diseases of humans and are characterized by intracellular infection and slowly evolving granulomatous lesions resulting in extreme tissue destruction.

M. leprae was the first bacterium shown to be associated with human disease. It appears to be extremely adapted to humans; it has not been successfully cultivated on artificial media and this failure makes it an extremely difficult organism to study and has hindered vaccine development. Fortunately, it is not highly contagious. M. tuberculosis has been cultivated in vitro and is pathogenic for other animals. Recently, a variety of other Mycobacterium species have been implicated in human diseases.

The usual immunologic response to mycobacterial infections in humans is a delayed-type hypersensitivity (DTH) which is the basis for diagnostic tests. This DTH response is characteristic of cell-mediated responses, the main mode of immunity. Increased resistance to infection in test animals can be passively transferred with

viable lymphocytes but not with serum. Humoral immunity plays little if any role in protection and is currently not of diagnostic importance. Contributing to the ineffectiveness of humoral immunity is the fact that mycobacterial species infect and multiply within macrophages.

Due to the recent success in cultivating M. leprae in limited quantities in armadillos, investigators have isolated M. leprae DNA. A variety of M. leprae specific antigens have been identified and cloned recently. (Young, R. et al., Nature 316: 450 (1985)). Several of these antigens have been shown to stimulate M. leprae specific T cell clones (Ottenhoff et al., Nature 319:66 (1986); Mustafa et al., Nature 319:63 (1986)).

Pox viruses of which vaccinia is the prototype are extremely large, complex DNA viruses which replicate solely within the cytoplasm of an infected host cell. Almost two hundred years ago, Edward Jenner proved that cowpox was an effective and safe vaccine to protect against smallpox. Vaccinia virus (similar to cowpox) has subsequently been used for the worldwide eradication of smallpox. Its effectiveness in the smallpox eradication program was due to relative safety, ease of use, stability, ease of administration and low cost.

Of all of the viruses which infect animals, pox viruses have several characteristics which uniquely suit them for use as eukaryotic expression vectors for generating new recombinant live vaccines. (U.S.

Patent No. 4,603,112: Modified Vaccinia Viruses. See also, Panicali, et al., Proc. Natl. Acad. Sci. USA 80: 5364 (1983); Smith et al. , Proc. Natl. Acad. Sci. USA 80: 7155 (1983); Cremer, et al., Science 228: 737 (1985).

The majority of recombinant pox viruses have contained genes coding for either viral or nonbacterial parasitic antigens.

Disclosure of the Invention

This invention pertains to recombinant pox viruses capable of expressing mycobacterial antigens, particularly mycobacterial antigens capable of eliciting a protective immune response against mycobacteria, to methods of producing the recombinant pox virus, to intermediate DNA vectors which recombine with pox virus in vivo to produce the modified pox viruses and to methods of vaccinating a host with the recombinant pox virus to elicit protective immunity against mycobacteria in the host. This invention also relates to the uses of mycobacterial antigens expressed by these recombinant vaccinia viruses in cultured cells as vaccines or for diagnostic reagents. This invention also pertains to a method of expressing foreign proteins as the result of gene fusions with endogenous vaccinia (poxvirus) genes.

Recombinant pox virus capable of expressing mycobacterial antigens are produced by integrating into the pox virus genome a gene or genes encoding

the mycobacterial antigen(s) of interest. The mycobacterial gene is inserted into a region of the pox virus genome which is nonessential for replication of the pox virus. The gene is inserted into the pox virus genome in association with a pox virus promoter to direct its expression.

The mycobacterial gene is integrated into the pox viral genome by an in vivo recombination event between an intermediate DNA vector carrying the mycobacterial gene and pox virus. In essence, the intermediate DNA vector contains the mycobacterial gene linked to a pox viral promoter located within a DNA sequence homologous to a region of the pox viral genome which is nonessential for replication of the pox virus. Thus, the vector comprises:

a. a prokaryotic origin of replication;

b. a pox viral promoter;

c. a gene encoding a mycobacterial antigen under the direction of the pox viral promoter;

d. DNA sequences of the pox virus into which the gene encoding the mycobacterial antigen is to be integrated. These DNA sequences flank the promoter and structural gene at both the 5' and 3' end and are homologous to the region of the pox virus genome where the mycobacterial gene is to be inserted.

In situations where the mycobacterial gene of interest contains a translational start signal which is not recognized as such by eukaryotic cells (e.g. GTG), the vector can be designed to contain a fused gene construct to allow for expression of the

mycobacterial antigen. This can be accomplished by linking, in proper reading frame, the mycobacterial gene containing the translational start signal to a DNA sequence containing an ATG start signal. For example, the mycobacterial gene can be linked to a pox gene or portion thereof which contains an ATG translational start signal recognizable by the eukaryotic host. This linkage can be made via a DNA linking sequence. In such embodiments, the fused gene encodes a tripartite fusion protein comprised of the mycobacterial protein with amino acids encoded by the pox gene and the DNA linker attached at its N terminus.

Recombination of the DNA vector and the pox virus is achieved in an appropriate host cell. Appropriate host cells for in vivo recombination are eukaryotic cells which are 1) transfectable by the DNA vector and 2) infectable by pox virus. The host cell is infected with the pox virus and then is transfected with the DNA vector. Virus is allowed to replicate in the host cell and recombination occurs in vivo resulting in insertion of the mycobacterial gene into the pox virus genome. The viral progeny is isolated away from the wild type virus. When a selectable marker has been co-integrated with the mycobacterial antigen, expression of the selectable marker provides a basis for selection of recombinant virus containing integrated mycobacterial DNA. Other methods of selection include detection of integrated

mycobacterial gene by hybridization with homologous DNA probes or selection for absence of the product of the viral gene into which the DNA vector has been inserted.

The recombinant virus is a virus which expresses in tissue culture and in an inoculated host the mycobacterial antigen of interest. The virally-expressed antigen will trigger cell-mediated and humoral immunity against the mycobacterium from which the antigen is derived. Moreover, certain antigens which are shared antigenic determinants may provide cross-protection against the various mycobacteria.

In preferred form, the DNA vector for recombination with pox virus also contains a gene which encodes a selectable marker which permits selection of viral recombinants containing the inserted mycobacterial DNA. Thus, the vector will contain these additional elements located between the flanking pox viral sequences:

e. a second pox viral promoter; and

f. a gene encoding a selectable marker, the gene being under the direction of the second pox viral promoter.

There are a number of advantages to creating a recombinant vaccinia virus expressing mycobacterial genes to use as a live vaccine. There is a long history of vaccinia virus being successfully and safely used in the eradication of smallpox. Vaccination with a live virus would also stimulate

cell-mediated and humoral immunity. The proteins that are expressed are expected to be appropriately modified, and if the required signals are present, they might also be localized to the proper regions of the cell or cellular membrane (Stephens et al., EMBO J 5:237 (1986)). In addition, it has been shown that vaccinia virus has the capability to recombine in at least 25Kb of DNA (Smith and Moss, Gene 25:21 (1983)); this amount of DNA can include a number of foreign genes encoding various antigens. These antigens could all be from the same organism or from a number of different organisms. Thus, a single vaccinia virus isolate could serve as a polyvalent vaccine against one or more pathogens (Perkus et al., Science 229:981 (1985)). Vaccinia virus vaccines can be manufactured inexpensively and the virus has been shown to be stable upon storage under normal field conditions. The vaccine can also be administered relatively simply. Finally, a recombinant vaccine utilizing vaccinia virus would avoid the problems associated with vaccinating with live attenuated or killed bacteria: these strains may not be properly killed or can become virulent. It is expected that live viral vaccines would also be more immunogenic than subunit vaccines.

Brief Description of the Figures

The Figures 1-4 show schematically the construction of the plasmid pAbT6007, a plasmid containing the 65K antigen of M. leprae which

recombines with unmodified vaccinia virus in vivo to yield a modified vaccinia virus which can express the 65Kd antigen (in the form of a fusion protein).

Figure 1 shows the construction of plasmid pAbT6000 which contains the gene encoding the 65 Kilodalton (Kd) antigen of M. leprae.

Figure 2 shows the construction of plasmid pDP502 which contains the BamF promoter of vaccinia and the lacZ gene adjacent thereto.

Figure 3 shows the construction of plasmid pAbT402 which contains, in order, thymidine kinase (TK) sequences of vaccinia, the vaccinia promoter for the 11Kd antigen of vaccinia and a portion of the 11K gene, the BamF-lacZ construct from pDP502 and additional TK sequences.

Figure 4 shows the final steps in construction of pAbT6007-the insertion of the gene for the 65K antigen of M. leprae (from pAbT6000) downstream of the 11K gene portion via a linker in proper frame with the 11K promoter.

Figure 5 shows the DNA sequence at the junctions of fusion of the 11K gene, the linker DNA and the gene for the 65K antigen of M. leprae and shows the predicted amino acid sequence at the junctions.

Figure 6 shows the construction of plasmids pAbT8000 and pAbT8005, precursors for construction of pAbT8008.

Figure 7 shows the construction of plasmid pAbT8008 which contains the vaccinia 7.5K promoter driving expression of the gene for the tuberculosis

65Kd protein and the Bam F promoter driving expression of the lacZ gene.

Figure 8 shows the construction of plasmid pAbT8032 which contains the gene for the leprosy 65Kd antigen driven by the vaccinia 7.5K promoter.

Figure 9 shows the construction of plasmid pAbT8501 which contains the gene for the leprosy 12Kd antigen under control of the 7.5K promoter of vaccinia.

Detailed Description of the Invention

1. Genes encoding mycobacterial antigens

Genes encoding mycobacterial antigens can be obtained from the genomic DNA of a mycobacterium or from available clones containing the genes. As mentioned, the isolation and cloning of M. leprae DNA has been described. See Young, et al., Nature 316:450 (1985), the teachings of which are incorporated by reference herein. Procedures for obtaining the DNA from other mycobacteria, e.g. M. tuberculosis and M. bovis, are reported by Clarke-Curtiss et al., J. Bact. 161:1093 (1985). Genes for tuberculosis antigens have aslo been cloned and analyzed (Young et al. Proc. Natl. Acad. Sci. USA 82:2583 (1985); Husson and Young, Proc. Natl. Acad. Sci. USA 84:1679 (1987); and Shinnick, J. Bact. 169:1080 (1987)).

Because cell-mediated responses appears to be the primary immunological responses involved in

protection against mycobacterial infection, preferred mycobacterial antigens are those which elicit a cell-mediated response in addition to a humoral (antibody) response. The 65Kd, 12Kd, 36Kd and 18Kd antigens of M. leprae, the 65Kd and 19Kd antigen of M. tuberculosis and the 65Kd of M bovis appear to be involved in the cell-mediated response to these mycobacteria. See e.g. Ottenhoff et al., Nature 319:66 (1986); Mustafa et al. Nature 319:63 (1986); Emmrich et al. Abstract, International Cong. of Immunol. (1986); Oftung et al., J. Immunol. 138:927 (1987); Emmrich et al., J. Exp. Med. 163:1024 (1986). In addition, mycobacterial antigens can be modified to enhance presentation of the antigen on the surface of virally infected cells. This can be accomplished by genetically engineering signal and anchor polypeptide sequences on the antigen. Specific mycobacterial epitopes (Mehra et al., Proc. Natl. Acad. Sci. USA 83:7013 (1986); Lamb et al., EMBO J. 6:1245 (1987)) can also be expressed on the cell surface with the appropriate signal and anchor sequences.

Especially preferred antigens are those antigens which are crossreactive between mycobacteria. For example, the 65Kd antigens of M. leprae, M. tuberculosis, and M. bovis have a significant degree of homology (Husson and Young, Proc. Natl. Acad. Sci. USA 84:1679 (1987)). Antigens of this type may provide cross-protective immunity against various mycobacteria.

2. Pox viruses

A suitable pox virus for generating recombinant virus includes any members of the pox family which do not cause significant disease in normal humans or animals. The preferred pox virus is vaccinia virus, a relatively benign virus which has been used for years as a vaccine against small pox. General techniques for integration of heterologous DNA into vaccinia virus to provide modified vaccinia virus capable of expressing foreign protein encoded by the heterologous integrated DNA are described by Paoletti et al. U.S. Patent No. 4,603,112, the teachings of which are incorporated by reference herein.

3. Preparation of intermediate DNA vectors for recombination with pox virus

According to the method of this invention, cloned mycobacterial genes which code for antigenic proteins are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by the pox virus along with the expression of the normal complement of pox virus proteins. This is accomplished by first constructing a prokaryotic insertion vector which contains (i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host, (ii) DNA sequences homologous to the region of the pox virus genome where the mycobacterial gene is to be inserted, (iii) the mycobacterial gene inserted at a site

within this pox virus sequence and (iv) a pox virus regulatory sequence (promoter) adjacent to the 5' end of the mycobacterial gene, constructed in a manner to allow for transcription of the mycobacterial gene and subsequent expression. This exact construction may contain only the vaccinia promoter sequence or the promoter and adjacent regions of the pox virus gene from which the promoter was derived. The viral promoter and virally-derived flanking sequences are obtained from genomic DNA or cloned fragments. The mycobacterial gene is obtained as described above. The vector backbone can be derived from any of several plasmid vectors capable of replication in a prokaryotic host, e.g., pBR322 (Sutcliffe, Cold Spring Harbor Symp, Quant. Biol. 43:77 (1979)), pUC8 (Vieira and Messing, Gené 19:259 (1982) or pEMBL (Dente et al., Nucleic Acids Res. 11:1645 (1982)). The ability to replicate in a prokaryotic host provides a means for amplification of the vector to produce sufficient quantities for transfection of a eukaryotic cell for recombinations.

In addition, the vector preferably contains a gene which encodes a marker which will allow selection of recombinant pox viruses containing integrated mycobacterial DNA. The gene encoding the marker is placed under control of a pox virus promoter. Several types of marker genes can be used. A preferred marker gene is the lacZ gene which encodes the enzyme beta-galactosidase. Re-

combinant pox virus will express beta-galactosidase along with the mycobacterial antigen and beta-galactosidase production is detected as an indication of insertion and expression of the foreign DNA. Recombinant viruses which express beta-galactosidase will form blue plaques while wild-type virus not expressing beta-galactosidase will form clear plaques. Recombinant viruses which have sequences inserted into their TK gene will grow in the presence of BUdR while virus containing a wild-type uninterupted TK gene will be unable to do so (Dubbs and Kit, Virol. 22:214 (1964); Smith et al., Proc. Natl. Acad. Sci. USA 80:7155 (1983)). Other selectable markers include genes which confer antibiotic resistance in an infected host cell, e.g., the Neo^R gene. Infected cells are grown in media containing the antibiotic at a concentration toxic for antibiotic sensitive cells. Under these conditions, cells infected with a recombinant virus expressing the resistance marker will produce virus. Cells infected with virus not containing the marker will not produce virus.

A preferred DNA vector for recombination with the preferred pox virus vaccinia virus comprises:

a. a vaccinia promoter (e.g., the vaccinia 11K, 7.5K, 30K, 40K or BamF promoter or modified versions of these promoters) linked to;

b. a structural gene encoding a mycobacterial antigen of interest (e.g., the 65Kd

antigen of M. leprae or M. tuberculosis) under control of the promoter;

c. a second vaccinia promoter (e.g., the BamF promoter of vaccinia virus) linked to;

d. a gene encoding a selectable marker (e.g., the lacZ gene); and

e. DNA sequences homologous with a region of vaccinia nonessential for replication flanking the construct of elements a-d (e.g., vaccinia thymidine kinase sequence).

Some prokaryotic genes contain the translational initiation codon GTG which is not functional in eukaryotic systems. This is the case, for example, for the gene encoding the 65Kd antigen of M. leprae. In some cases, the cloned gene may not have an initiation codon because it is incomplete and therefore one must be supplied. In such instances, the intermediate vector can be designed so that the prokaryotic gene is linked to an ATG codon. For example, this can be done via a sequence containing an ATG. Preferably, this is accomplished by constructing a fused gene containing a promoter of pox virus and the adjacent coding regions of the pox virus gene from which the promoter is derived. The promoter and ATG initiation codon can be linked to the prokaryotic gene by a DNA linker. In this case, the fused gene construct would encode a tripartite fusion protein and the prokaryotic antigen would be expressed as such. The tripartite

gene product would comprise (from N-terminus to C-terminus):

a. amino acids encoded by vaccinia ATG-supplying DNA segment;

b. linker encoded amino acids; and

c. prokaryotic protein.

The fused gene can be designed to minimize the number of amino acids of nonmycobacterial origin so as not to alter significantly immunogenic or other properties of the mycobacterial antigen. Alternatively, if it is inconvenient to use the ATG initiation codon which was originally associated with the promoter of interest, one can choose a linker sequence which will provide an in-frame ATG. In such a case, the recombinant protein would be comprised of amino acids encoded by the linker DNA and the mycobacterial gene.

As described in more detail in the examples below, the gene for the 65Kd antigen of M. leprae was inserted into an intermediate vector containing a) the vaccinia thymidine kinase gene, as the sequence for homologous recombination, b) the promoter and codons specifying the first two amino acids of the 11K gene of vaccinia virus, to direct expression of the 65Kd antigen, and c) the E. coli B-galactosidase gene under the control of the vaccinia Bam F promoter, to facilitate identification of the recombinant virus by use of a chomogenic assay. Using this intermediate vector, a recombinant vaccinia virus was created which expresses the 65Kd antigen. The vaccinia expressed

65Kd antigen was recognized by two monoclonal antibodies which recognized specific epitopes on the 65Kd antigen.

4. In vivo recombination

The intermediate DNA vectors containing the mycobacterial gene (and the marker gene) flanked by appropriate pox viral sequences will undergo recombination with pox virus genomic DNA which results in integration of the flanked gene into the viral genome. Recombination will occur in a eukaryotic host cell. Appropriate host cells for recombination are those which are 1) infectable by pox virus and 2) transfectable by the DNA vector. Examples of such cells are chick embryo fibroblast, CV-1 (monkey kidney cells), HuTK 143 cells (human cells), BSC40 (monkey kidney cells).

Viral infection is accomplished by standard techniques for infection of eukaryotic cells with pox virus.

The cells can be transfected with the intermediate vector by any of the conventional techniques of transfection. These include the technique of calcium phosphate precipitation, DEAE dextran, electroporation and protoplast fusion. The preferred technique is the calcium phosphate precipitation technique.

After infection and subsequent transfection, the cells are incubated under standard conditions and virus is allowed to replicate, during which time in vivo recombination occurs between the homologous

pox virus sequences in the intermediate vector and the pox virus sequences in the genome.

Recombinant viral progeny are then selected by any of several techniques. The presence of integrated mycobacterial DNA can be detected by hybridization with a labeled DNA probe specific for the mycobacterial DNA. Alternatively, virus harboring the mycobacterial gene can be selected on the basis of inactivation of the viral gene into which foreign DNA was inserted. For example, if the DNA vector is designed for insertion into the TK gene, viruses containing integrated DNA will be TK^- and can be selected on this basis. Preferred techniques for selection, however, are based upon co-integration of a gene encoding a marker or indicator gene as described above. The preferred indicator gene is the LacZ gene. Selection of recombinant viruses expressing B-galactosidase can be done by employing a chromogenic substrate for the enzyme.

5. Vaccine compositions

Recombinant pox viruses expressing mycobacterial antigens can be used as live viral vaccines to vaccinate hosts (humans or animals) susceptible to mycobacterial disease. Recombinant pox viruses expressing cross-protective antigens such as the 65Kd antigen of M. leprae, M. tuberculosis or M. bovis may provide cross protective immunity in both humans or animals. In other instances, it may be necessary to use recombinant viruses expressing one

or more type specific mycobacterial antigens to induce sufficient immunogenic response in the vaccinated host to be protective.

Recombinant pox viruses may be produced in cultured eukaryotic cells. Other techniques may be used to produce pox virus vaccines e.g. production of vaccinia virus in calves.

6. Diagnostic Applications

The mycobacterial antigens produced during the infection with recombinant pox virus are also of diagnostic value. For example, during recombinant pox infection, the M. leprae 65Kd antigen can be produced; this antigen reacts with monoclonal antibodies to M. leprae 65Kd antigen to an extent which can be detected by ELISA. This result shows the feasibility of using M. leprae antigens produced in pox infected cells for testing for the presence of M. leprae-specific antibodies and T cells in humans. This can determine whether or not individuals have been exposed to M. leprae.

The invention is illustrated further by the following Examples.

EXAMPLES

MATERIAL & METHODS

E. coli Strains:

E. coli strains JM101 (Messing et al., Nucl. Acids Res. 9:309 (1981)), MC1060 (Casadaban & Cohen, J. Mol. Biol. 138, 179 (1980)) and RR1 (Bolivar et al., Gene 2:95 (1977); Peacock et al., Biochem. Biophys. Acta 655:243 (1981) were used.

Restriction Enzyme Digestion:

Enzymes were obtained from New England Biolabs or Boehringer-Mannheim. Digests were performed as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982, Molecular Cloning; A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 104-105). Digests were incubated at 37°C for 1 hr unless otherwise specified.

Treatment of DNA with Calf Intestinal Phosphatase:

DNA was dephosphorylated in 50mM Tris-HCl, Tris-HCl, pH9.0, 1.0mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine with 1ul of calf intestinal phosphatase (CIP) (Boehringer Mannheim, 23 units/ul) at 37°C for 30 min, sometimes followed by a second 30 min incubation with another 1ul of enzyme.

Treatment of DNA with DNA Polymerase, Large Fragment (Klenow):

Klenow enzyme was obtained from New England Biolabs and used as described (Maniatis, T.,

Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 112-113).

Gel-Purification of Restriction Fragments:

DNA was fractionated by size on low-melt agarose (ranging from 0.7% to 3%) gels run in 40mM Tris-acetate pH8.0, 2mM EDTA. The DNA fragment of interest was excised from the gel, liquified at 70°C and diluted in 200mM NaCl, 50mM Tris, pH7.5, 1mM EDTA. The DNA was extracted with phenol, then phenol:chloroform (1:1) and precipitated with ethanol.

Isolation of DNA from Agarose Gels using Glass Powder:

The DNA fragment of interest was excised from an agarose gel. The slice was placed in an Eppendorf tube, weighed and dissolved at 60°C in 2-3 ml of NaI solution (6.06M NaI which had been saturated with 1.5g of Na_2SO_3 and then filtered) per gm of gel. After the agarose was dissolved, 2-10 ul of glass slurry (Vogelstein and Gillespie, Proc. Natl. Acad. Sci. USA 76:615 (1979)) was added and allowed to bind to the DNA for 30-60 min on ice. The following steps were then performed at 4°C:

1. The centrifuge tube was spun for 15 sec to pellet glass, and the supernatant was removed.
2. The glass pellet was resuspended in 1 ml of NaI solution.

3. The tube was again centrifuged for 15 sec and the resulting supernatant was discarded.

4. The pellet was resuspended in 1 ml of ethanol wash solution (50% ethanol, 0.1M NaCl, 10 mM Tris-HCl pH 7.5) which had been stored at -20°C.

5. The glass suspension was centrifuged and then supernatant was removed.

6. Steps 4 and 5 were repeated.

Next the pellet was resuspended in 50-100 ul of TE and the DNA was eluted at 37° for 30-60 min. The suspension was centrifuged and the supernatant containing DNA was removed to a new tube. The pellet was resuspended in another 50-100 ul of TE and spun down and this second supernatant was combined with the first one. The DNA was then extracted once with phenol/chloroform, twice with chloroform, and then precipitated with ethanol. The DNA was resuspended in TE.

Ligation of DNA Fragments:

T4 DNA Ligase was obtained from Boehringer Mannheim. Ligations were performed in 50mM Tris-HCl, pH7.4, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM spermidine, 1mM adenosine 5'-trisphosphate, 0.1mg/ml bovine serum albumin, 1u T4 DNA ligase, at 15°C for 30 min to 3 days unless otherwise specified.

Phosphorylation of Linkers and Ligation to DNA:

Linkers and T4 polynucleotide kinase were obtained from New England BioLabs. Linkers were phosphorylated and ligated as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 396-397).

E. coli Transformation:

E. coli cells were made competent and transformed with DNA as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y., pp 250-251).

Isolation and Purification of Plasmid DNA:

Preparation of plasmid DNA and purification by cesium chloride-ethidium bromide gradient centrifugation were performed as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 90, 91, 93, 94).

Virus and Cells:

CV-1 cells, which are kidney cells derived from the African Green Monkey, were obtained from the American Type Culture Collection (ATCC #CCL70) and grown in Minimal Essential Media (MEM; Gibco) supplemented with 10% fetal calf serum (FCS; Hyclone

Laboratories, Inc.). BSC40 cells were obtained from B. Roberts (Harvard Medical School, Boston, MA) and were grown in DME and 10% FSC.

Vaccinia virus strain NYCBH (New York City Board of Health) was obtained from the ATCC (catalog #ATCC VR-325). The virus stock received from ATCC was grown on CV-1 cells in MEM-2% FCS, and this virus was further amplified in spinner cultures of HeLa-S3 cells (ATCC #CCL2.2) for purification on sucrose gradients and infection in in vivo recombination (IVR) experiments.

Infection and Transfection:

CV-1 cells were plated 24 hr before an IVR at 10^6 cells per 6cm plate. These cells were infected with vaccinia virus at a multiplicity of infection (MOI) of 0.05 to 2 in a total volume of 200ul of MEM-2% FCS. Virus was adsorbed for 40 min at 37°C; during this time the plates were rocked every 10-15 min to distribute the virus and to keep the plates from drying out. After virus adsorption, 3.3ml of MEM-2% FCS was added to the cells.

Solution A (250mM $CaCl_2$, 25mM HEPES, pH7.12) was added to 20ug of CsCl gradient-purified DNA (total volume of 30ul or less) for a final total volume of 250ul. While air was then bubbled through this solution, 250ul of solution B (280mM NaCl, 1.5mM Na_2HPO_4, 25mM HEPES, pH7.12) was added dropwise, and the solution was incubated at room temperature for 40 min. This solution was then

added dropwise to infected cells while the plate was gently swirled.

The infected cells which had been transfected with DNA right after completion of virus adsorption were incubated at 37°C until all the cells were observed to be infected; typically, cytopathic effects were seen by 16-20 hr. Virus from the IVR was harvested by freezing and thawing the plates three times. Cells were scraped off the plate into the medium, cell debris was removed by centrifugation in a clinical centrifuge for 5 min, and the supernatant containing the virus was sonicated for 40 sec or vortexed and stored at -80°C.

Plaque Purification of Recombinant Virus:

The progeny virus was then titered on a monolayer of CV-1 cells on a 6cm plate. Because the DNA used in the transfection step contained the gene for lacZ under the regulation of a vaccinia promoter as well as a gene of interest, i.e. gene encoding antigen, one could detect recombinant virus as blue plaques in the presence of Bluo-Gal (Bethesda Research Laboratories). Because in vivo recombination occurs at a low frequency and blue plaques could be isolated from no more than 250-300 background clear plaques, the virus titer from each IVR was determined by a titration curve in which the virus yield was diluted 10^{-1} through 10^{-4} in MEM-2% FCS.

A 6cm plate of confluent CV-1 cells (passaged 24 hr earlier) was infected with 0.5ml of diluted virus which was adsorbed for 30 min at 37°C. The medium was then removed by aspiration and the cells were overlaid with 3ml of Dulbecco's Modified Eagle's Medium (DME) minus Phenol Red (Gibco) - 0.6% agarose (Bio-Rad) - 10% FCS - 0.1ug/ml fungizone. This overlay was made by mixing equal volumes of 2x (DME minus Phenol Red-FCS-fungizone) with 1.2% agarose. On day 3, another 3ml of overlay containing the previous mixture and 400ug/ml Bluo-Gal (from a freshly prepared stock at 20mg/ml in dimethyl sulfoxide (DMSO)) was placed over the first agarose overlay. The plates were then screened daily for the number of blue and white plaques; blue plaques first appeared anywhere from 4-48 hr.

Having calculated the plaque forming units/ml (PFU) for the progeny virus in the IVR, one can now set up an infection of 15-20 6cm plates of CV-1 cells and use that amount of virus calculated to result in 250-300 plaques per plate. This infection, covering of cells with first and second overlay and screening of the plaques were all done as described above. Recombinant blue plaques were picked and placed into 2.0ml MEM-2% FCS and sonicated for 40 sec or vortexed several times. A next round of plaque purification was done by infecting cells with 20ul to 200ul of this sonicated virus,

and this was followed by a third and fourth round of plaque purification.

Virus Amplification:

After plaque purification, the resulting virus was amplified on a 6cm plate of CV-1 cells. This virus was harvested in a total volume of 5ml and the cells lysed by 3 cycles of freeze-thawing. Cellular debris was removed by centrifugation and the resulting virus was again amplified first on another 6cm plate of CV-1 cells, and then again on a 15cm plate of CV-1 cells. The virus particles (in 20ml) were then concentrated by layering over a 15ml cushion of 36% (w/v) sucrose in 1mM Tris-HCl, pH9.0, and centrifuging in the SW28 rotor at 20,000 rpm at 4°C for 60 min. The virus pellet was resuspended in a small volume of 1mM Tris-HCl, pH9.0. The concentration of PFU was determined as previously described; in this case dilutions ranged from 10^{-3} to 10^{-8}.

The virus resulting from the infection of the 15cm plate of CV-1 cells was then used to infect approximately 1 liter of HeLa-S3 cells in a spinner at an MOI of 1. Virus was trypsinized with an equal volume of 0.25% trypsin (Gibco) for 30 min at 37° C in a shaker. HeLa-S3 cells were spun down, resuspended in 20ml Dulbecco's phosphate-buffered saline (DPBS: 137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 4.9mM $MgCl_2$, 9.1mM $CaCl_2$, pH7.4) and counted; the cells were then further concentrated by

centrifugation and resuspended in a small volume (approximately 2ml) of DPBS-1% FCS. The appropriate amount of trypsinized virus was added to the cells and adsorbed for 45 min in a shaker at 37°C. The infected cells were then diluted with fresh DME-10% FCS to a final concentration of approximately 4 x 10^5 cells/ml. After a 3 day incubation at 37°C, the cells were pelleted, resuspended in approximately 100ml of 1mM Tris-HCl, pH 9.0, and left on ice for 30 min. The cells were then homogenized in 25ml aliquots with 10 strokes each in a Dounce homogenizer. The cells were pelleted at 6,000 rpm in the Sorvall SA-600 rotor for 5 min and the supernatant containing the virus was set aside. The pelleted cells were resuspended in 20 ml of 1mM Tris-HCl, pH9.0, and homogenized again with five strokes of a Dounce homogenizer. The cells were pelleted at 8,000 rpm in the Sorvall SA-600 rotor and the supernatant containing the virus was pooled with the first supernatant. At this point, the virus was frozen at -80°C until further purification through a sucrose cushion and a sucrose gradient. Virus was purified by pelleting through a 15 ml cushion of 36% (w/v) sucrose in 1mM Tris-HCl, pH 9.0, at 20,000 rpm at 4°C for 60 min in the SW28 rotor. The virus pellet was resuspended in a small volume of 1mM Tris-HCl, pH 9.0, and banded on a 25-40% sucrose (w/v) gradient in 1mM Tris-HCl, pH 9.0, in a SW28 rotor at 15,000 rpm at 4°C for 40 min. The virus band was collected and repelleted at

20,000 rpm in the SW28 rotor for 60 min at 4°C. The pellet was resuspended in 1mM Tris-HCl, pH 9.0 and subsequently titered.

RNA Dot Blots:

Cells were infected at an MOI of 2 and grown for 16-24 hr. The medium was removed, cells were washed twice with PBS and 600 ul lysis buffer (equal volumes of DMSO mixed with an equal volume of 4 M guanidinium thiocyanate, 25mM sodium citrate (pH7), 0.5% sarkosyl, 0.1M B-mercaptoethanol was added. The cells were incubated at 37°C for 30 min and then scraped and collected into Eppendorf tubes. The samples were stored at -20°C.

The samples were filtered through nitrocellulose on a dot blot manifold (Schleicher and Schuell) and washed twice with of 200ul 20XSSC (3M NaCl, 0.3M Na citrate) and once with 2XSSC. The filter was air-dried, and baked at 80°C in a vaccum oven.

The filter was prehybridized for 2 hr and hybridized overnight in 50% formamide, 3XSSC, 5X Denhardts (1 X Denhardts is 1% Ficoll, 1% polyvinylpyrrolidone, 1% Bovine Serum Albumin), 0.1% SDS and 100ug/ml tRNA at 42°C. The ^{32}p-labeled probe added to the hybridization mix was generated by oligolabeling (OLB; Feinberg and Vogelstein, Anal. Biochem. 137:266 (1984); Feinberg and Vogelstein, Anal. Biochem. 132:6 (1983)) with the Pharmacia Oligolabeling Kit (#27-9250).

Following hybridization, the filter was washed twice for 30 min in 3XSSC at room temperature and then twice for 30 min in 0.1X SSC at 65°C. After air-drying, the filter was exposed to film for 2 days with a screen at -70°C.

RNA Preparation for Hybrid Selections:

CV-1 cells were infected with vaccinia virus and harvested at various times during infection. The cells were washed twice in cold PBS which was then removed. The cells were then lysed with ice-cold 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7), 0.5% Sarkosyl, and 0.1M beta-mercaptoethanol. The cell lysate was then passed at least four times through a syringe needle to shear DNA, layered onto a 3-ml cushion of 5.7 CsCl-0.1 M EDTA treated with diethylpyrocarbonate, and centrifuged for 18 h at 15°C in an SW50.1 rotor at 35,000 rpm. The RNA pellet was washed with 70% ethanol, suspended in water, and precipitated with ethanol after the addition of 0.1 volume of 2 M sodium acetate.

Hybrid Selection of RNA:

One ug of linearized denatured plasmid DNA was spotted onto nitrocellulose filters which were then baked in a vaccum oven at 80°C for 2 hr. The filters were then incubated with 40 ug RNA in 65% formamide, 0.01 M Pipes, pH 6.4, 0.4 M NaCl for 3 hr at 48°. Unhybridized RNA was removed by ten washes

of 1X SSC and 0.5% SDS at 57°C followed by three washes with 2.5 mM EDTA. Hybridized RNA was released by boiling the filter for 60 sec in 1 mM EDTA. This RNA was recovered, precipitated with 10 ug tRNA and trnslated in a rabbit reiculocyte cell-free translation system.

Cell-free Translation of RNA:

Rabbit reticulocyte lysate was obtained from Promega Biotec and translation reactions with ^{35}S methionine were done according to their directions. The translation products were analyzed on SDS polyacrylamide gels (Laemmli, Nature 227: 680 (1970)) and processed for fluorography using Enchance (New England Nuclear).

Black Plaque Assay:

CV-1 cells were plated out one day prior to infection at $7x10^5$ cells per 6cm dish, in MEM containing antibiotics and antimycotics (Sigma) and 10% FCS. Twenty-four hours later the cells became confluent ($1x10^6$ cells per dish). Growth medium was removed from the cells and replaced with 0.5ml of medium containing the recombinant vaccinia virus (approximately 100 PFU/plate) or control virus (NYCBH). Virus was adsorbed to the cells for 30 minutes at 37°C, and at the end of this time, the medium was removed by aspiration and the cells were overlaid with 0.6% agarose in DME containing 10%FCS

and 0.1ug/ml fungizone. The cells were then incubated for two days to allow plaques to form.

After the two day incubation, the agarose overlay was removed, leaving the cell monolayer intact on the dish. The cells were washed three times with 3ml of DPBS, and were fixed for 15 min at room temperature, using 3% formaldehyde (Mallinckrodt) in phosphate-buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 1.5 mM KH_2PO_4, 8.1mM Na_2HPO_4). The monolayer was again washed three times with 3ml of DPBS, and was then incubated with a primary antibody, e.g. IIC8 (Gillis and Buchanen, Infect. Immunity 37, 172 (1982)) or Y1-2 (Engers et al., Infect. Immunity 48:603 (1985)). These two monoclonal antibodies both received as ascites fluid, were obtained from Dr. Richard Young, Whitehead Institute for Biomedical Research, Cambridge, Ma). The monoclonal IIC8 was diluted 1:1,270 in 50% normal goat serum (NGS; Colorado Serum Company) in PBS, and 1ml of this solution was added to the monolayer, and incubated overnight at 4°C with constant shaking. When monoclonal antibody Y1-2 was used, it was diluted 1:500 in 50% NGS, and 1ml of this solution was incubated with the monolayer for 1 hr at 37°C. After the incubation, the antibody solution was removed by aspiration and the monolayer was washed three times with 3ml of washing buffer (20mM Tris-HCl, pH 7.5, 1M NaCl, 0.05% Tween-20). The cells were then incubated with the secondary antibody, alkaline phosphatase-conjugated

goat anti-mouse IgG (heavy and light chain specific; Kirkegaard & Perry Laboratories). This antibody was diluted 1:200 in 10% NGS, and 1ml was incubated with the monolayer for 1 hr at 37°C. The solution was then removed by aspiration, and the cells were washed three times with 3ml of washing buffer, and once with Tris-buffered saline (TBS; 20mM Tris-HCl, pH7.5, 0.15 M NaCl). Color was developed on plaques which were expressing the antigen of interest by incubating with a precipitating substrate system, BCIP/NBT (5-bromo-4-chloro-3indolyl-phosphate and nitroblue tetrazolium (Kirkegaard & Perry Laboratories)). The reagent was made up as per manufacturer's instructions, and 2ml was incubated with the monolayer at room temperature until color developed on the plaques (approximately 30 min).

Live Black Plaque Assay on BSC40 Cells:

Dishes containing 6 wells were coated with 0.1% gelatin in PBS. The plates remained at room temperature for two hr after which the gelatin was removed and the plates were air-dried in the hood.

BSC40 cells (5 X 10^5/3.5cm well) were plated and the following day the cells were infected with approximately 100 PFU of virus. The virus was adsorbed for 30 min at 37°C and then removed. Four ml of DME - 2% FCS (heat inactivated) were added. Two days later, the live black plaque assay was done.

The live black plaque assay was done under sterile conditions. The medium was removed from the cells and the plates were washed three times with DPBS. The plates were incubated at room temperature with the primary antibody, Y1.2, at a 1:500 dilution in 3% BSA in DPBS. The first antibody was then removed and the plates were washed three times with DPBS. The second antibody (.5ml), an alkaline-phosphatase-conjugated goat anti-mouse IgG, was diluted 1:1000 in 3% BSA in DPBS was added to the plate which was incubated for 1 hr at room temperature. The second antibody was removed and the cells were washed twice with DPBS and once with TBS. In order to develop color on those plaques expressing the leprosy 65Kd antigen, a BCIP/NBT mix (Kirkegaard and Perry) was added to each plate. Plaques which were positive for antigen expresion were picked with a sterile toothpick into 2 ml of DME-2% FCS.

Enzyme-Linked Immunosorbance Assay (ELISA):

CV-1 cells were infected at an MOI of 2. The cells were grown to confluence and infected as described in the previous section, except that the 0.5ml of medium containing virus was left on the cells after the 30 minute incubation, and 4.5ml of fresh medium was added. The cells were then incubated for 18 hours at 37°C.

Following the incubation period, the medium was aspirated from the cell monolayer, and the cells were gently scraped off the dish into 1ml of DPBS.

This mixture was frozen and thawed (-80°C to 37°C) three times, and then sonicated. The treated cell pellets were used to coat microtiter plates to supply antigen on a solid phase for ELISA.

Immulon II remove-a-well strips (Dynatech) were coated in duplicate with 200ul of the cell pellet for 18 hrs at room temperature. The solution was then removed by aspiration, and 200ul of primary antibody (e.g. Y1-2 monoclonal diluted 1:500) in 50% NGS was added to the wells. A negative control antibody, A_4E_7, was also used. This monoclonal antibody (in ascites fluid) recognized an epitope on the unrelated canine parvovirus. The primary antibody was incubated for six hr at 37°C. This solution was removed by aspiration and the wells were washed three times with 0.5ml of washing buffer (0.05% Tween-20 in PBS, pH7.5). The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chain specific; Jackson Immunoresearch) diluted 1:2,500 in 10% NGS. It was incubated at 200ul per well for 1 hr at 37°C. Following the incubation, the solution was removed by aspiration and the wells were washed three times, as described above. Color was developed using 3,3', 5,5'-tetramethyl-benzidine (TMB,Sigma) as the chromagen. Ten milligrams of TMB were dissolved in 1ml of DMSO, and 100ul of this solution was added to 5ml of acetate/citrate buffer (0.1M sodium acetate, pH6.0 with 0.1 M citric acid) along with 10ul of 3% H_2O_2 (Park-Davis); 200ul of this final solution was

added to the wells and the plate was incubated at room temperature for 5 min. The reaction was stopped by the addition of 200ul of 2.5 N H_2SO_4. One half of the solution in each well was removed, and the remaining 200ul was read at 450nm on the Dynatech Mini-Reader II plate reader.

Metabolic Labeling:

CV-1 cells were grown for 24 hr to a density of 10^6 cells per 6cm plate and then infected with vaccinia virus at an MOI of 2 for 30 min at 37°C. Four hours later, the cells were labeled with [^{35}S]methionine. The labeling medium consisted of 2ml of methionine-free DME, 3.5% FCS, 2mM L-glutamine, 100uCi [^{35}S]methionine (New England Nuclear) and carrier methionine (0.3mg/100ml). Alternatively, cells were labelled with [^{3}H] leucine (New England Nuclear) in 2 ml of leucine-free DME, 3.5% FCS, 2mM L-glutamine, approximately 100 uCi [^{3}H] leucine and carrier leucine (1.05 ug/ml). Cells were harvested after approximately 20 hr, washed twice with PBS, and lysed with 0.5ml of immunoprecipitation buffer (IPB: 10mM Tris-HCl, pH7.2, 650mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5mM EDTA, 1mM phenylmethylsulfonylfluoride, and 0.1mg/ml trypsin inhibitor) for 10 min at room temperature. For subsequent immunoprecipitation steps, 1mM ATP was also included in the buffer used. The lysates were stored at -80°C.

Preparation of Staphylococcus Aureus:

Killed and formalin-fixed S. aureus (IgSorb) were obtained as a 10% (w/v) suspension from the Enzyme Center (Malden, Ma). The cells were washed twice with IPB and pelleted by spinning 5 min in a microfuge. The cells were resuspended to a final concentration of 10% or 20% (w/v) in IPB.

Preformed complexes of S. aureus and IgG were prepared by incubating five volumes of 10% (w/v) S. aureus with one volume of anti-vaccinia virus serum for 10 min at 0°C. The complexes were pelleted, washed twice, and resuspended to a final concentration of 20% (w/v) in IPB.

Immunoprecipitation:

Immunoprecipitations were carried out on cell lysate samples each containing 10uCi [^{35}S]methionine in 0.2ml of IPB. All incubations with antibodies or S. aureus were done with rocking at 4°C. Each sample was precleared by incubating with 50ul of performed immune complex (S. aureus-IgG, 20% [w/v]) for 1 hr and then clarifying by centrifugation in the microfuge for 5 min. The supernatant was transferred to a new test tube, and the preclearing was repeated. Each of the pellets was saved and processed as described below. The resulting supernatant was then treated for another 30 min with 50ul of 20% (w/v) S. aureus. Finally, the supernatant was transferred to a new test tube and incubated with primary antibodies at an appropriate dilution.

The next day, 50ul of 10% (w/v) S. aureus were added to the sample, and the incubation was continued for 30 min. The S. aureus complexes were pelleted by centrifugation. Each pellet (including those from the preclearing steps) was washed with 200ul of IPB. The suspension was layered over a differential sucrose gradient (400ul of 10% (w/v) sucrose/0.5 M NaCl/IPB, plus 800ul of 30% (w/v) sucrose/0.14 M NaCl/IPB) and spun for 10 min in the microfuge. The resulting precipitate was washed with 200ul of IPB and resuspended with 20ul of SDS gel sample buffer (Laemmli, Nature 227; 680 (1970)). The sample was boiled for 5 min, centrifuged to remove the precipitate, and removed with a syringe for application onto an SDS gel (Laemmli, U.K. 1970, Nature 227, 680) which contained an 8% separation gel and a 3% stacking gel. The SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions and was followed by autoradiography of the gel.

Immunoprecipitations Using Protein A-Sepharose in Place of Preformed Complexes of S. Aureus - IgG:

^{3}H leucine - labeled lysates were precipitated with trichloroacetic acid (TCA) to determine the amount of radioactivity in each lysate. Antiserum was added to an aliquot (1 uCi of TCA-precipitable counts) of each sample and this mixture was then gently agitated at 4°C for 2 hr to overnight. Protein A-Sepharose was pre-swollen, washed twice

with IPB and 50 ul in IPB (1:1;v/v) was added and the mixture was gently agitated for 1 hr at 4°C. The Protein A-Sepharose was washed 4 times at 4°C with 1 ml of IPB by spinning for 15 sec in a microfuge to pellet the Protein A-Sepharose. The Sepharose was then washed once with 1 ml of 0.01 M Tris-HCl, pH8.2, 150 mM NaCl at 4°C. The pellets were dried and resuspended in 20-40 ul of 1X Laemmli sample buffer and prepared for analysis on SDS polyacrylamide gels.

Immunization of mice:

Adult female Balb/c mice (Taconic Labs) were immunized intraperitoneally with 10 PFU of vaccinia recombinants expressing mycobacterial antigens.

Serum was obtained from the vetro-orbital plexus on a weekly basis for four weeks and was assayed for the presence of anti-mycobacterial antibodies on nitrocellulose filter lifts of recombinant lambda phage.

Adsorption of mouse antiserum with an E. coli lysate:

In order to remove nonspecific binding of mouse antisera to E.coli proteins, mouse antisera was adsorbed on an E.coli lysate coupled to Affi-Gel (Bio-Rad).

A lysate of E.coli strain Y1090r-m^+ was made by growing a one liter culture at 37°C to an A_{600} of approximately 0.5. The bacteria were pelleted by

centrifugation at 4200 Xg for 10 min at 15°C. The supernatant was discarded and the pellet resuspended in 20 ml of PBS. This was frozen in liquid nitrogen and then thawed at room temperature to generate the lysate. The preparation was sonicated for 30 seconds and 7mls was dialyzed (in a 3500 molecular weight cut-off membrane) against 1 liter of 0.01 M bicarbonate buffer, pH 9.6, with three changes of buffer.

Ten mls of both Affi-Gel 10 and Affi-Gel 15 (Bio-Rad) were mixed and were washed with 5 volumes of ice-cold H_2O in a scintered glass funnel. This gel was then added to the dialyzed lysate and the mixture was incubated at room temperature for three hours with constant gentle agitation. The gel was then pelleted by centrifugation, and washed three times with TBS. The gel was stored in an equal volume of 0.002% NaN_3 in TBS.

Mouse antisera was cleared of anti-E.coli reactivity by incubating 0.1ml of mouse antiserum plus 0.9 ml of 10% NGS with 0.4 ml of the immunoadsorbent described above. It was incubated with gentle mixing overnight at 4°C, and the immunoadsorbent pellet was then removed by centrifugation. This pre-cleared mouse serum was then tested for antibody to mycobacterial antigens on nitrocellulose filter lifts.

Screening Antisera on recombinant lambda phage:

Y1090r-m+ was grown overnight to saturation in LB which contained 100ug/ml ampicillin. Fifty microliters (200 PFU) of recombinant phage diluted in 10mM Tris-HCl, pH7.5, 10mM $MgCl_2$, was added to 100ul of the Y1090 culture. This was incubated for 20 min at 37°C to allow phage to adsorb to the bacteria. At this time 1.25 ml of soft agar equilibrated at 48°C was added to each tube containing the bacteria and phage. The tubes were swirled gently and poured onto 6cm LB agar plates. The cultures were incubated for 3.5 hr at 42°C. They were then overlayed with nitrocellulose filters saturated in IPTG, and incubated for 3.5 hours at 37°. The filters were carefully removed from the dishes, and were blocked overnight at 4°C in 10% NGS in PBS.

The blocking solution was aspirated, and adsorbed mouse antiserum, diluted 1:10 in 50% NGS was added and incubated for 1 hr at room temperature. The filters were washed three times in washing buffer (20mM Tris-HCl, pH7.5, 0.05% Tween, 1M NaCl) and were then incubated with alkaline phosphatase labelled goat anti-mouse IgG (Kirkegaard and Perry Labs) diluted 1:1000 in 10% NGS. After 1 hr at room temperature, the filters were again washed, and were then incubated with a BCIP/NBT substrate solution (Kirkegaard and Perry Labs) according to manufacturers instructions.

EXAMPLE 1

Construction of Plasmids pAbT2008 and pAbT6000 (Figure 1)

pUC8 (Vieira and Messing, Gene 19:259 (1982)) was partially digested with HaeII, and the 2,258 base pair (bp) fragment was gel-purified and religated to create pAG3 as shown in Figure 1A.

pAbT2008 was a modification of pAG3 in that it contained an EcoRI site at what was once the NdeI site. It was created by digesting pAG3 with NdeI, filling in the staggered DNA ends with Klenow enzyme, ligating on EcoRI linkers (d(pGGAATTCC); New England BioLabs), removing excess linkers by EcoRI digestion, and religating the resulting DNA fragment (Figure 1B). pAbT2008 was grown in the E. coli strain JM101.

The gene encoding the 65Kd antigen of Mycobacterium leprae was contained within an EcoRI DNA fragment cloned into lambda gt11. This lambda clone was originally obtained from Dr. R. Young (Whitehead Institute for Biomedical Research, Cambridge, MA) and was designated lambda Y3178 (Young et al., Nature 316:450 (1985)). This DNA was digested with EcoRI, and the 3.6 Kb EcoRI DNA fragment containing the M. leprae gene was purified from an agarose gel. It was ligated to plasmid pAbT2008 which had been digested with EcoRI and treated with CIP, resulting in plasmid pAbT6000 (Figure 1C). This DNA was transformed into E. coli

strain GM119, a dam$^-$ E. coli strain, to yield unmethylated plasmid DNA.

EXAMPLE 2

Construction of pDP502 (Figure 2):

Plasmids pMC1871 and pSKS107 (Shapira et al., Gene 25:71 (1983)) were obtained from M. Casadaban (The University of Chicago; Chicago, Ill.) These plasmids contained the E. coli lacZ gene flanked by various restriction endonuclease sites.

Preparative amounts of pMC1871 were digested to completion with SstI (SacI). Approximately 10ug of this DNA was partially digested with BamHI and the fragments were separated on a preparative agarose gel. A 5,380bp band was isolated from this gel. This fragment was missing the 5' end of the lacZ gene from the BamHI site to the unique SacI site found within the lacZ gene (Figure 2A).

Preparative amounts of pSKS107 were digested to completion with SacI and BamHI. The DNA fragments were then separated on an agarose gel and a 2,040bp fragment was isolated. This fragment contained the 5' end of the lacZ gene from a BamHI site to the SacI site (Figure 2A).

Appropriate quantities of these fragments (5,380bp and 2,040bp) were then ligated to each other using standard ligation conditions. The resulting ligated DNA was used to transform

competent E. coli cells, strain RR1. This plasmid was designated pMC1871-7 (Figure 2B)

Preparative amounts of pMC1871-7 were digested with BamHI to completion, the fragments were then separated on an agarose gel and a fragment of approximately 3000bp was isolated. This fragment contained the entire coding region of the lacZ gene except for the five 5' most codons which were deleted in the original pSKS107 construction (a polylinker from a pUC plasmid was inserted in place of these sequences in the original construction). The 5' end of the lacZ gene had the following predicted sequence: GA TCC GTC GAC CTG CAG CCA AGC TTG GCA. The last amino acid codon GCA coded for alanine which was the sixth amino acid in the native lacZ polypeptide.

Plasmid pRW120 (Panicali et al., Proc. Natl. Acad. Sci. USA. 80:5364 (1983)) contained a PstI fragment subcloned from the HindIII F fragment of vaccinia virus (strain WR) DNA; the PstI fragment spanned a unique BamHI site in the HindIII F fragment. The PstI fragment was inserted into the plasmid vector pBR325 (Bolivar et al., Gene 2:95 (1977)) from which the vector BamHI site had been removed. The vaccinia BamHI site was adjacent to a vaccinia transcription promoter region which had been used to direct the expression of several foreign genes inserted at the BamHI site. These foreign genes were inserted such that their orientations were consistent (in the same relative

orientation for transcription) with the vaccinia gene.

A preparative amount of pRW120 was digested to completion with BamHI and then treated with CIP. This DNA was ligated to the BamHI lacZ fragment from pMC1871-7 and the resulting DNA was used to transform competent E. coli RR1 cells. This plasmid was designated pDP502 (Figure 2C).

To determine if the coding region of the lacZ gene was in frame with the vaccinia gene at the BamHI site, pDP502 was sequenced through the junction of the 5' end of the lacZ insert and the adjacent vaccinia DNA. This was done using standard dideoxy sequencing techniques (Wallace et al., Gene 16:21 (1981)) directly on pDP502, using a commercially available kit and M13 lacZ sequencing primer (Amersham). The sequence analysis indicated that the lacZ gene was in frame with the ATG of the vaccinia promoter sequence (Figure 2D), allowing for expression of B-galactosidase.

EXAMPLE 3

Construction of Plasmids pAbT400, pAbT401 and pAbT402 (Figure 3):

pAG3 was digested with NdeI and treated with the large fragment of DNA polymerase (Klenow) to blunt the ends of the fragment. The vaccinia thymidine kinase gene, located on the vaccinia HindIII J fragment (Weir et al., Proc. Natl. Acad.

Sci. USA. 79:1210 (1982); Hruby and Ball, J. Virol. 43:403 (1982); Weir and Moss, J. Virol. 46:530 (1983)), was subcloned into pBR322. The thymidine kinase gene was excised from this clone by digesting with HindIII and PvuII, producing an 1,800bp fragment which was gel-purified and treated with Klenow to blunt the ends of the fragment. The two fragments described above were ligated to create pAbT400, in E. coli strain MC1060, as shown in Figure 3B.

pAbT400 was digested with ClaI and EcoRI and was treated with CIP. The vaccinia 11K gene was located on the vaccinia HindIII F fragment (Wittek et al., J. Virol, 49:371 (1984); Bertholet et al., Proc. Natl. Acad. Sci. USA 82:2096 (1985)), which had been subcloned into pBR322 (Panicali and Paoletti, Proc. Natl. Acad. Sci. USA 79:4927 (1982)). This plasmid was digested with ClaI and EcoRI, producing a 600bp fragment which was gel-purified. The two fragments described above were ligated to create pAbT401, in E. coli strain MC1060, as shown in Figure 3C.

pAbT401 was digested with EcoRI and treated with Klenow to produce a blunt-ended fragment; BglII linkers (d(pCAGATCTG); New England BioLabs) were ligated to the ends of the fragment and excess linkers were removed by BglII digestion. pDP502 was first digested with BglII and then partially digested with BamHI; the resulting 3,900bp BglIIBamHI fragment was gel-purified. The two fragments

described above were ligated to create pAbT402, in E. coli strain MC1060, as shown in Figure 3D.

EXAMPLE 4

Construction of Plasmid pAbT6007 (Figure 4):

A 2,353bp DNA fragment containing the coding region for the M. leprae 65Kd antigen was excised from pAbT6000 by digestion with NruI and EcoRV. Bg1II linkers (d(pGGAAGATCTTCC); New England Bio-Labs) were ligated onto this DNA, excess linkers were removed by Bg1II digestion, and the approximately 2,360bp DNA fragment was isolated from a gel (Figure 4A).

The plasmid pAbT402 was digested with Bg1II, treated with CIP and then ligated to the 2,360bp DNA fragment. This DNA was used to transform E. coli JM101, and the resulting recombinant plasmid DNA containing the M. leprae 65Kd gene linked to the vaccinia 11K late promoter was designated pAbT6007 (figure 4B). This plasmid also contained the E. coli lacZ gene regulated by the vaccinia BamF (Panicali and Paoletti, Proc. Natl. Acad. Sci. USA, 79:4927 (1982); Panicali et al., Proc. Natl. Acad. Sci. USA. 80:5364 (1983)) promoter. Flanking the lacZ gene and the M. leprae gene were vaccina thymidine kinase sequences. Cesium chloride-purified pAbT6007 DNA was prepared for IVR into vaccinia virus strain NYCBH in CV-1 cells.

It should be noted that there was a translation initiation codon (ATG) on the DNA fragment containing the 11K promoter. Because the Bg1II linker added onto the 2,360bp DNA fragment put this ATG in the same reading frame as the translation initiation codon (GTG) of the M. leprae 65Kd protein, the recombinant vaccinia virus containing this DNA should express a fusion protein that has its first nine amino acids specified by the nucleotide sequence from the 11K promoter, the Bg1II linker and those M. leprae nucleotides which precede its first GTG (Figure 5). In addition, the GTG is expressed as a valine in vaccinia whereas in M. leprae it is the translation initiation codon and thus is expressed as methionine.

EXAMPLE 5

Recombinant Vaccinia Virus Containing the leprosy 65Kd Antigen by In Vivo Recombination:

In vivo recombination is a method whereby recombinant vaccinia viruses are created (Nakano et al., Proc. Natl. Acad. Sci. USA. 79: 1593 (1982)). These recombinant viruses are formed by transfecting DNA containing a gene of interest into cells which have been infected by wild type vaccinia virus (in this case NYCBH). The genes that one wants recombined into the vaccinia genome are flanked on both sides by vaccinia DNA sequences which serve to direct the recombination of exogenous DNA to the

site where these vaccine sequences are found. A small percent of the progeny virus will contain the gene of interest integrated into a specific site on the vaccinia genome. These recombinant viruses can express genes of foreign origin (Panicali and Paoletti, Proc. Natl. Acad. Sci. USA, 79: 4927 (1982); Panicali et al., 1983, Proc. Natl. Acad. Sci, USA, 80: 5364 (1983)).

pAbT6007 DNA was transfected into vaccinia virus-infected CV-1 cells at an MOI of 2. The virus strain was NYCBH and the selection system for recombinant virus was the appearance of blue plaques due to the metabolism of Bluo-Gal directed by the lacZ gene. Approximately $1x10^6$ CV-1 cells on a 6cm plate were infected for 40 min at 37°C. After adsorption, 3.3ml of MEM-2% FCS was added to the plate. For the calcium phosphate precipitation of DNA, 243.2ul of Buffer A was added to 20ug of pAbT6007 DNA (originally at a concentration of 2.95mg/ml), followed by the addition of 250ul of Buffer B, as already described. After 40 min, this DNA was added dropwise to the infected cells, which were then incubated at 37°C until 100% of the cells were observed to be infected.

The infected cells and virus were harvested and the virus was titered as previously detailed. The 10^{-2} dilution resulted in 250-300 plaques per plate and twenty 6cm plates of CV-1 cells were each infected with 0.5ml of the 10^{-2} dilution of virus obtained from the IVR. Several blue plaques were

picked from these plates, and after four cycles of plaque purification, four final blue plaques were picked and amplified twice on 6cm CV-1 plates. Two of these were amplified on 15cm plates and concentrated by centrifugation through a 36% sucrose cushion as already described. One isolate, vAbT53-1-1-1-2 was picked for further amplification in a HeLa-S3 spinner culture. It was designated vAbT53. The virus was centrifuged through a 25-40% sucrose gradient in a SW28 rotor at 15,000 rpm at 4°C for 40 min. This preparation yielded virus at a final concentration of 1.36×10^9 PFU/ml.

EXAMPLE 6

Black Plaque Assay for the Leprosy 65Kd Antigen Expression:

The black plaque assay is an in situ enzyme-based immunoassay which can detect protein expressed by cells, both uninfected and those infected by virus.

The black plaque assay was performed on the recombinant vaccinia vAbT53, which contained the gene for the leprosy 65Kd antigen, as well as on wild-type vaccinia lacking this gene. Monoclonal antibodies IIC8 or Y1-2 were used.

Plaques formed by the negative control, NYCBH virus, showed only a background color which was consistent with the background on the cell monolayer itself. Plaques formed by the vaccinia recombinant

however, stained a distinct dark purple color which was much darker than the background on the cell monolayer. These results were obtained with both antibodies, and showed that the recombinant virus vAbT53 was expressing the M. leprae antigen.

EXAMPLE 7

Enzyme-Linked Immunosorbance Assay (ELISA) for the Leprosy 65Kd Antigen Expression:

The results are summarized in Table 1; monoclonal antibody Y1-2 recognized an antigen in vAbT53 but not in the uninfected cells or in cells infected with NYCBH virus. The monoclonal A_4E_7 did not recognize antigens in infected or uninfected cells. These results showed that vABT53 was expressing the M. leprae 65Kd antigen.

TABLE 1

Results* of ELISA on Vaccinia Recombinant vAbT53 Expressing the 65Kd M. Leprae Antigen.

Monoclonal Antibody	Specificity	Cells Infected with: vAbT53	NYCBH	No Infection
Y1-2	65 kd antigen of M. leprae	1.28	0.60	0.49
A_4E_7	canine parvo-virus	0.13	0.12	0.13

* Results are expressed as the average of duplicate O.D. readings at 450nm on the ELISA plate reader.

EXAMPLE 8

Immunoprecipitation of Leprosy 65Kd Antigen From Vaccinia Recombinant vAbT53:

Immunoprecipitation analysis was performed on CV-1 cells infected with recombinant vaccinia virus vAbT53 encoding the 65Kd protein of M. leprae. Cells were labeled with [^{35}S]methionine for approximately 20 hr. The cell lysates were precleared of background proteins using an anti-vaccinia virus serum and then incubated with a mouse monoclonal antibody IIC8 (dilution 1:200) directed against the 65Kd protein. Antibodies were precipitated with S. aureus, and the crossreacting antigens were detected by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Each of the three vaccinia recombinant samples tested incorporated radioactivity into a 68Kd protein. This signal was absent in cells infected with wild-type vaccinia virus or with recombinant virus containing other foreign genes. Background proteins that were derived from vaccinia-encoded genes were identified on the basis of their cross-reactivity with the anti-vaccinia virus serum.

EXAMPLE 9

Construction of pAbT8000 and pAbT8005 (Figure 6)

Lambda phage Y3253 (Husson and Young, Proc. Natl. Acad. Sci. USA 84:1679 (1987)) which contained the gene encoding the tuberculosis 65kd antigen, was obtained from Dr. R. Young of the Whitehead Institute of Biomedical Research, Cambridge, MA. Phage DNA was prepared and then digested with EcoRI. This EcoRI DNA fragment of approximately 2.1kb contained the gene for the tuberculosis 65Kd antigen and was purified from an agarose gel. It was ligated to pEMBL18 DNA (Dente et al. Nuc. Acids Res. 11:1645 (1982)) which had been digested with EcoRI and treated with CIP. The resulting plasmid, shown in Figure 6, was designated pAbT8000.

DNA from pAbT8000 was then digested with the restriction enzymes BspMII and KpnI. BspMII cleaves the DNA just upstream of the initiating ATG codon. The DNA was then blunt ended with T4 DNA polymerase. BglII linkers (New England Biolabs #1052) were ligated onto the blunt-ended DNA. The addition of this BglII linker served to place the ATG contained within the 11K promoter fragment into frame with the ATG of the gene encoding the 65Kd antigen. The DNA was digested with BglII and a fragment of about 1.8Kb was purified from a gel and ligated to pAbT402 DNA which had been cut with BglII and treated with CIP. The ligated DNA was used to transform HB101 E.coli cells, and minipreparations of plasmid DNA

were purified and digested with various restrcition enzymes to isolate a plasmid in which the gene for the tuberculosis 65Kd antigen was in correct orientation relative to the 11K promoter of pAbT402.

This resulting plasmid was designated pAbT8005 (Figure 6) and contains the vaccinia 11K promoter driving the expression of the gene for the tuberculosis 65Kd antigen. This 11K - 65Kd cartridge is followed by the vaccinia Bam F promoter controlling the expression of the lacZ gene. These two promoters and their genes are flanked by the vaccinia TK gene which serves to direct in vivo homologous recombination into the vaccinia TK gene.

EXAMPLE 10

Construction of pAbT8008 (Figure 7)

pAbT8005 DNA (Figure 7), which contains the gene for the tuberculosis 65Kd antigen, was digested with BglII and then blunt-ended with Klenow. A fragment of approximately 1.8 Kb was then purified from an agarose gel and ligated to pAbT4007 DNA which had been digested with SmaI and then treated with CIP. pAbT4007 is a vaccinia expression vector (See U.S. Patent application Serial No. 910,501, - Panicali, Mazzara & Gritz filed September 23, 1986) which contains a 7.5K promoter to drive the expression of an inserted foreign gene. As in pAbT402, the pAbT4007 vector contains a downstream vaccinia BamF promoter driving lacZ expression. The

ligation mixture consisting of pAbT4007 DNA and the BglII fragment from pAbT8005 was used to transform E. coli HB101 cells. Minipreparations of DNA were purified and the plasmids were analyzed by restriction digests to determine which DNAs contained the BglII fragment in the proper orientation for the expression regulated by the 7.5K promoter. This DNA was then designated pAbT8008.

pAbT8008 contains the vaccinia thymidine kinase gene flanking the following.

1. the vaccinia 7.5K promoter driving the expression of the gene for the tuberculosis 65Kd protein.
2. the Bam F promoter driving the expression of the gene for lacZ.

EXAMPLE 11

Construction of pAbT8032 (Figure 8)

DNA from pAbT6007 (Figure 4B) was digested with BglII to generate a 2352bp fragment which contained the coding sequence for the gene for the leprosy 65Kd protein. The DNA was then blunt-ended with Klenow, and Cla linkers (New England Biolabs #1037) were ligated on. The Cla linker provided an ATG which was in frame with the coding sequence of this leprosy gene, and was needed because the 7.5K promoter lacked an ATG and the initiation codon on this mycobacterial gene was GTG, instead of the usual ATG. The DNA was then cleaved with ClaI and

fractionated on an agarose gel. The 2,360 fragment was purified and ligated to pBR322 DNA that had been digested with ClaI and treated with CIP. The ligated DNA was transformed into E. coli HB101 and a clone containing the ClaI insert was designated pAbT8031 (Figure 8).

pAbT8031 DNA was digested with ClaI and treated with Klenow to blunt the ends. The DNA was fractionated by size on an agarose gel, and the 2,360 bp fragment containing the gene for the leprosy 65Kd antigen linked to an in-frame ATG was ligated to pAbT4007 DNA which had been cut with SmaI and then treated with CIP. A clone containing the gene for the leprosy 65Kd antigen in the correction orientation for expression regulated by the 7.5K promoter was isolated and designated pAbT8032 (Figure 8).

pAbT8032 contains the gene for the leprosy 65Kd antigen preceded by an in-frame ATG which serves as the initation codon. This gene is controlled by the vaccinia 7.5K promoter and is followed by the BamF promoter driving the expression of the lacZ gene. These elements are then flanked by the vaccinia thymidine kinase gene.

EXAMPLE 12

Construction of pAbT8023, pAbT8502 and pAbT8501 (Figure 9)

Lambda clone Y3184 (obtained from R. Young, Whitehead Institute contains the gene encoding a protein which exhibits an affinity to a biotin-conjugated antibody for a leprosy 12Kd antigen. The DNA encoding the antigen was sequenced (R. Young, personal communication) and found to be incomplete. The predicted amino acid sequence of this protein has homology to several proteins which are known to bind biotin (e.g. Maloy et al., J. Biol. Chem. 254:11615 (1979)) and it is possible that this is the reason the lambda clones reacted postively to the biotin-conjugated antibody. The DNA sequence predicts a polypeptide whose minimum size is 18Kd, and based upon the size of other biotin-binding proteins, this may be close to the full-length size. For purposes of continuity, this DNA is still referred to as the gene for the leprosy 12Kd antigen.

DNA from lambda clone Y3184 was isolated and digested with EcoRI. This EcoRI fragment was then cloned into pEMBL18 DNA which had been digested with EcoRI and treated with CIP. The ligated DNA was transformed into E. coli strain HB101, and a plasmid containing the 2.4Kb EcoRI insert was designated pAbT8023 (Figure 9).

pAbT8023 DNA was digested with EcoRI and then blunted with Klenow. Cla linkers (New England Biolabs #1089) were ligated onto the blunt-ended DNA which was then digested with ClaI. Because this leprosy gene was not a full-length sequence, an in-frame ATG was needed, and we have provided one with this Cla linker. The 620bp Cla-linkered fragment was purified from a gel and ligated with pBR322 DNA that had been cut with ClaI and treated with CIP. The ligated DNA was transformed into HB101 cells, and minipreparations of DNA were prepared and analyzed by restriction digests for isolation of a clone which contained a 620bp ClaI DNA fragment. This clone was designated pABT8502 (Figure 9).

pAbT8502 DNA was digested with ClaI and then blunt-ended with Klenow. The 620bp fragment was cloned into the SmaI site of pAbT4007. This DNA ligation was transformed into HB101 cells. The DNA prepared from various HB101 colonies was analyzed by restriction endonuclease analysis in order to isolate a plasmid which contained the sequence for the leprosy 12Kd antigen in the correct orientation for expression from the 7.5K promoter. This DNA was designated pAbT8501 (Figure 9).

pAbT8501 contains the gene for the leprosy 12Kd antigen under the control of the 7.5K promoter and this cartridge is followed by the BamF promoter driving the lacZ gene. The vaccinia thymidine kinase gene flanks these two foreign genes.

EXAMPLE 13

Recombinant Vaccinia Virus Containing the Leprosy 65Kd Antigen (vAbT121), Tuberculosis 65Kd Antigen (vAbT86, vAbT88) or the Leprosy 12Kd Antigen (vAbT117).

vAbT121 was created by infecting cells CV-1 cells with NYCBH at an MOI of 0.05 and transfecting in 15 ug of linearized pAbT8032 (original concentration of 1.33 mg/ml) DNA. The IVR was harvested at 48 hr and then titered. The recombinant was purified through four rounds of plaque purification on BSC40 cells grown in DME. In the first, third and fourth rounds, recombinant plaques were picked by their blue phenotype in the presence of Bluo-gal and IPTG. The determination of recombinant plaques in the second round of purification was done by the live black plaque assay wherein the primary antibody was the Y1.2 mouse monoclonal and the second antibody was an alkaline phosphatase conjugated goat anti-mouse IgG. The final isolate vAbT121C-B-1L-1-1 was designated vAbT121 and amplified in a Hela-S3 spinner culture. This virus expressed the leprosy 65Kd antigen during early and late infection under the control of the 7.5K promoter.

vAbT86 which expresses the tuberculosis 65Kd antigen under the control of the 11K promoter during late infection, was produced by infecting CV-1 cells with NYCBH at an MOI of 1, and then transfecting in 20 ug of pAbT8005 plasmid DNA (original

concentration 1.48 mg/ml). The virus from the IVR was harvested after 24 hr and the recombinants were purified through four rounds of plaque purification on CV-1 cells. In each round, the expression of the lacZ gene indicated the recombinant plaques. A final isolate, vAbT86-2-2-1-1 was picked for further amplication and designated vAbT86.

vAbT88 was generated by infecting CV-1 cells with NYCBH at an MOI of 1, and transfecting in 20 ug of pAbT8008 plasmid DNA (original concentration of 4.7 mg/ml). The virus resulting from the IVR was harvested after 24 hr. Recombinants were purified through four rounds of plaque purification by lacZ expression on CV-1 cells. A final isolate, vAbT88b-2-1-1-1, expressed the tuberculosis 65Kd antigen during early and late infection, was designated vAbT88. Expression of this antigen was under the control of the 7.5K prompter.

vAbT117 was prepared by infecting CV-1 cells with NYCBH and transfecting in 20 ug of pAbT8501 plasmid DMA (original concentration 2.9 mg/ml). The virus resulting from this IVR was harvested at 48 hr and recombinants were purified through three rounds of plaque purification on CV-1 cells. Recombinants were selected by expression of the lacZ gene at each round of purification. One isolate, vAbT117-18-1-4-1 was designated vAbT117 and was amplified on Hela-S3 cells. The characteristics of these four recombinant viruses are summarized in Table 2. Results of immunoprecipitation and animal studies are also tabulated here. The predicted

amino acid sequences encoded by these recombinant virus are shown in Table 3. It is expected that these proteins will not significantly differ from the proteins produced in mycobacteria.

EXAMPLE 14

Black Plaque Assay for Tuberculosis and Leprosy 65Kd Antigen Expression:

vAbT86, and vAbT88, and vAbT121 were tested by a black plaque assay for expression of the tuberculosis 65Kd antigen with the Y1-2 monoclonal antibody. NYCBH was used as a negative control. Plaques formed by the three vaccinia recombinants were stained a dark purple color while control plaques showed only a background color. A mycobacterial 65Kd antigen was being expressed by each recombinant virus.

EXAMPLE 15

ELISA for the Tuberculosis and Leprosy 65Kd Antigen Expression:

ELISA results for vAbT86, vAbT88 and vAbT121 are summarized in Table 4. Monoclonal antibodies Y1-2 and TB-78 recognized antigens in cells infected by these three recombinant viruses but not in uninfected cells or cells infected by the wild-type NYCBH virus. vAbT86 and vAbT88 were expressing the tuberculosis 65Kd antigen while vAbT121 was expressing the leprosy 65Kd antigen.

TABLE 2

SUMMARY OF RECOMBINANT VIRUSES AND THEIR IMMUNOGICAL RESULTS

Plasmid	Virus	Promoter	When Expressed	Antigen	ELISA Y1.2[2]	ELISA TB78[2]	Immunoprecipitations Strength and Molecular Weight Y1.2	Immunoprecipitations Strength and Molecular Weight TB78	Injected Into Mice	Anti 65k Antibc Detected
pAbT8005 pTK11KTB65K502	vAbT86	11K	Late	TB65K	++	++	Good 66K	Very Faint 66K	Yes	Yes
pAbT8008 pTK7.5KTB65K502	vAbT88	7.5K	Early & Late	TB65K	+	-	Strong 68K	Good 68K	Yes	Yes
pAbT6007 pTK11KL65K502	vAbT53	11K	Late	Leprosy 65K	++	ND[4]	Good 68K	Negative	Yes	Yes
pAbT8032 pTK7.5KL65K502	vAbT121	7.5K	Early & Late	Leprosy 65K	++	ND[4]	Good 68K	ND[4]	No	-
pAbT8501 pTK7.5KL12K502	vAbT117	7.5K	Early & Late	Leprosy 12K	ND[4]	ND[4]	ND[4]	ND[4]	Yes	Yes

Notes:

1. Don't know if this promoter is active during late infection.
2. Y1.2 monoclonal antibody recognizes leprosy 68K (L65K) antigen and is cross-reactive with the tuberculosis 65K (TB65K) antigen. The TB78 monoclonal antibody recognizes TB65K and is not cross-reactive.
3. Leprosy 12K expression also checked by RNA dot blot analysis and by hybrid selection.
4. Not done.
5. Antibodies in mice sera were tested by reaction with B-gal fusion proteins produced by lambda gt11.

TABLE 3

Predicted Amino Acid Sequences of Mycobacterial Proteins Encoded by Recombinant Vaccinia Viruses

Leprosy 65Kd Antigen:

Bacteria								Leprosy							
								C	GAT	CAG	CGA	GTG	CCA	GGT	CGG
												M	P	G	R
vAbT53	11K			Linker			Leprosy								
(pAbT6007)	ATG	AAT	TCA	GAT	CTT	CCC	GAT	CAG	CGA	GTG	CCA	GGT	CGG		
	M	N	S	D	L	P	D	Q	R	V	P	G	R		
vAbT121	7.5K		Linker	Linker			Leprosy								
(pAbT8032)	CCC	CCG	ATG	GAT	CTT	CCC	GAT	CAG	CGA	GTG	CCA	GGT	CGG		
			M	D	L	P	D	Q	R	V	P	G	R		

Tuberculosis 65Kd Antigen:

Bacteria								Tuberculosis							
								C	CGG	ACC	AAT	CAC	TTC	GCA	ATG	GCC	AAG	ACA
														M	A	K	T
vAbT86	11K			Linker			Tuberculosis											
(pAbT8005)	ATG	AAT	TCA	GAT	CTT	CCC	CGG	ACC	AAT	CAC	TTC	GCA	ATG	GCC	AAG	ACA		
	M	N	S	D	L	P	R	T	N	H	F	A	M	A	K	T		
vAbT88	7.5K			Linker			Tuberculosis											
(pAbt8008)	C	CCC	GAT	CTT	CCC	CGG	ACC	AAT	CAC	TTC	GCA	ATG	GCC	AAG	ACA			
												M	A	K	T			

Leprosy 12Kd Antigen:

Bacteria				Linker[1]			Leprosy								
				GAA	TTC	GAG	GTC	GAA	GGC	CTG					
				E	F	E	V	E	G	L					
vAbT117	7.5K	Linkers				Leprosy									
(pAbT8501)	CCC	CCG	ATG	GAA	TTC	GAG	GTC	GAA	GGC	CTG					
			M	E	F	E	V	E	G	L					

Notes

1. Linkers used in constructing lambda gt11 library.

TABLE 4

Results of ELISA on Vaccinia Recombinants vAbT86, vAbT88 and vAbT121.

Vaccina Recombinant	Antibody	ELISA[1] Recombinant	NYCBH	No Infection
vAbT86	TB78	1.83	0.25	N.D.[2]
vAbT88	Y1.2	1.61	0.25	N.D.[2]
vAbT121	Y1.2	0.61	0.10	0.06

Notes:

1. Results are expressed as the average of duplicate O.D. readings at 450nm on the ELISA plate reader.

2. Not done.

EXAMPLE 16

Immunoprecipitation of the Tuberculosis and Leprosy 65Kd Antigen:

CV-1 cells were infected with vAbT86, vAbT88, vAbT121 or NYCBH. Cells were labeled with ^{3}H leucine for approximately 20 hr. Cells were harvested and immunoprecipitations were done as described using protein A-Sepharose. The results are summarized in Table 2. All three recombinants express a 65 - 68Kd antigen which was not present in NYCBH.

EXAMPLE 17

Expression Analysis of the Leprosy 12Kd Antigen:

Because we were unable to obtain good monoclonals against this particular antigen, the expression analysis of vAbT 117 was accomplished by RNA dot blots, hybrid selection and generation of mouse antiserum to the original lambda gt11 clone Y3184. The hybridization with RNA dot blots showed that CV-1 cells infected with vAbT117 contained RNA which hybridized to a 620bp ClaI DNA fragment purified from pAbT8502. Hybrid selection showed that RNA obtained from CV-1 cells infected with vAbT117 hybridized to pAbT8502 plasmid DNA and this RNA was translated into a polypeptide of approximately 18-19 Kd. The RNA for the hybrid selection experiments was obtained from CV-1 cells

that had been infected at an MOI of 10; these cells were treated with cycloheximide, which was present during the entire infection (Mahr and Robert, J. Virol. 49:497 (1984).

Mice injected with the recombinant vAbT117 were bled and their sera were tested for the presence of antibodies to the lacZ fusion protein from Y3148. The results showed that the mouse sera contained antibodies to the polypeptide whose DNA sequence was contained within Y3148. This polypeptide is the 12Kd antigen.

EXAMPLE 18

Mouse Antisera Raised to Leprosy and Tuberculosis 65Kd Antigen:

Mice were vaccinated with 10 PFU of vAbT86, vAbT88 or vAbT53. The sera were collected at various times post inoculation and then tested for the presence of antibodies to the leprosy or tuberculosis 65Kd antigen. Lambda gt11 clone Y3178 was used as the antigen source for vAbT53. Clone Y3253 was the source of tuberculosis 65Kd antigen, the target of mouse antibodies generated by inoculating with vAbT86 and vAbT88. The results showed that vAbT86 and vAbT88 were able to raise mouse antibodies to the tuberculosis 65Kd antigen synthesized by E.Coli infected with phage Y3253. Vaccinia recombinant vAbT53 elicited mouse antibodies to the polypeptide synthesized by E.coli infected with Y3178.

Deposit of Biological Material

Plasmid pAbT6007 was placed on deposit at the American Type Culture Collection (ATCC) in Rockville, MD on September 5, 1986. Plasmids pAbT8005, pAbT8008, pAbT8032 and pAbT8501 were placed on deposit at the ATCC on September __, 1987.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such

equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A recombinant pox virus capable of expressing a mycobacterial antigen.

2. A recombinant pox virus of Claim 1, which is the species vaccinia.

3. A recombinant pox virus of Claim 1, wherein the mycobacterial antigen is an antigen of M. leprae, M. tuberculosis or M. bovis.

4. A recombinant pox virus of Claim 3, wherein the antigen is the 65Kd antigen of M. leprae.

5. A recombinant pox virus of Claim 3 wherein the antigen is the 12Kd antigen of M. leprae.

6. A recombinant pox virus of Claim 3 wherein the antigen is the 65Kd antigen of M. tuberculosis.

7. A recombinant vaccinia virus capable of expressing the 65Kd antigen of M. leprae or M. tuberculosis.

8. A recombinant vaccina virus of Claim 7 selected from the group consisting of vAbT86, vAbT88, vAbT53 and vAbT121.

9. A recombinant vaccinia virus capable of expressing the 12Kd antigen of M. leprae.

10. A recombinant vaccinia virus of Claim 9 comprising vABT117.

11. A recombinant pox virus capable of expressing a mycobacterial antigen which is capable of inducing immunity against mycobacteria.

12. A recombinant pox virus of Claim 11, which is of the species vaccinia.

13. A recombinant pox virus of Claim 11, wherein the mycobacterial antigen is an antigen of M. leprae, M. tuberculosis or M. bovis.

14. A recombinant pox virus of Claim 13, wherein the antigen is the 65Kd antigen.

15. A recombinant pox virus of Claim 11 wherein the antigen is the 12Kd antigen of M. leprae.

16. A plasmid DNA vector for in vivo recombination with a pox virus to produce a recombinant pox virus capable of expressing a mycobacterial antigen, comprising;

a. structural gene or portion thereof encoding a mycobacterial antigen;

b. a pox virus promoter linked to the structural gene and positioned so as to direct expression of the structural gene encoding the mycobacterial antigen;

c. DNA sequences of a pox virus flanking the promoter and structural gene at both 5' and 3' ends, the flanking pox sequences being sequences which allow recombination into a region of pox virus which does not inhibit the ability of the virus to replicate.

17. A plasmid of Claim 16, wherein the structural gene encodes a mycobacterial antigen of M. leprae, M. tuberculosis or M. bovis.

18. A plasmid of Claim 16, wherein the pox virus is of the species vaccinia.

19. A plasmid of Claim 16, wherein the promoter is selected from the group consisting of the vaccinia 11K, 7.5K, 30K, 40K and BamF promoters.

20. A plasmid of Claim 16, wherein the flanking sequences are derived from the gene of vaccinia virus which encodes thymidine kinase.

21. A plasmid of Claim 16, further comprising;

d. a second promoter of the pox virus linked to a second structural gene which encodes a marker or indicator for selection of recombinant pox virus, the second promoter and second structural gene being located between the flanking DNA sequences of the pox virus.

22. A plasmid of Claim 21, wherein the second promoter of the pox virus is the BamF promoter of vaccinia.

23. A plasmid of Claim 21, wherein structural gene which encodes a selectable marker is the lacZ gene.

24. A plasmid DNA vector for in vivo recombination with vaccinia virus to produce a recombinant vaccinia virus capable of expressing a mycobacterial antigen, comprising;

a. structural gene or portion thereof encoding an antigen of M. leprae, M. tuberculosis or M. bovis;

b. a first vaccinia promoter linked to the structural gene, the promoter being located upstream with the structural gene;

c. a lacZ gene;

d. a second vaccinia promoter linked to the lac Z gene, the promoter being located

upstream of and in proper reading frame with the lac Z gene; and

e. DNA sequences flanking the construct of elements a-d, the sequences being homologous with sequences of the vaccinia thymidine kinase gene.

25. A plasmid DNA vector of Claim 24, wherein the antigen is the 65Kd antigen.

26. A plasmid DNA vector of Claim 24 wherein the antigen is the 12Kd antigen of M. leprae.

27. A plasmid vector of Claim 24, wherein the first promoter is linked to the gene encoding the mycobacterial antigen through a region of the gene for the 11Kd antigen containing at least the translational start signal.

28. The plasmids pAbT8005, pAbT8008, pAbT6007, pAbT8032 and pAbT8501.

29. A method of preparing a recombinant pox virus capable of expressing a mycobacterial antigen, comprising the steps of:

a. providing a plasmid DNA vector comprising:

i. structural gene or portion thereof encoding a mycobacterial antigen;

ii. a pox virus promoter linked to the structural gene and positioned so as

to direct expression of the structural gene encoding the mycobacterial antigen;

iii. DNA sequences of a pox virus flanking the promoter and structural gene at both 5' and 3' ends, the flanking pox sequences being sequences which allow recombination into a region of pox virus which does not inhibit the ability of the virus to replicate;

b. allowing the plasmid vector to recombine with pox virus in vivo to produce recombinant pox virus; and

c. isolating the recombinant pox virus.

30. A method of Claim 29, wherein the pox virus is a vaccinia virus.

31. A method of Claim 29, wherein the structural gene encodes a mycobacterial antigen of M. leprae, M. tuberculosis or M. bovis.

32. A method of Claim 29, wherein the structural gene encodes the 65Kd antigen of M. leprae or M. tuberculosis.

33. A method of Claim 29, wherein the structural gene encodes the 12Kd antigen of M. leprae.

34. A method of Claim 29, wherein the promoter is the vaccinia 11K, 7.5K, 30K, 40K or BamF promoter.

35. A method of Claim 29, wherein the step of allowing the plasmid vector to recombine with the pox virus in vivo comprises:

i. infecting the host cell with pox virus;

ii. transfecting the infected cell with the plasmid vector; and

iii. incubating the cells under conditions which allow the virus to replicate.

FIGURE 1a

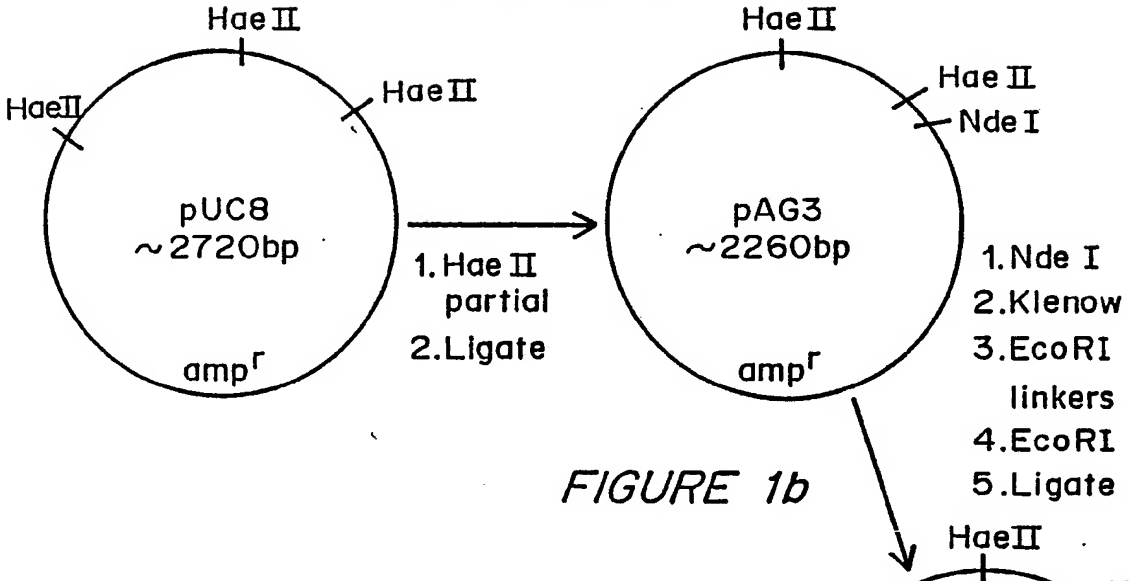
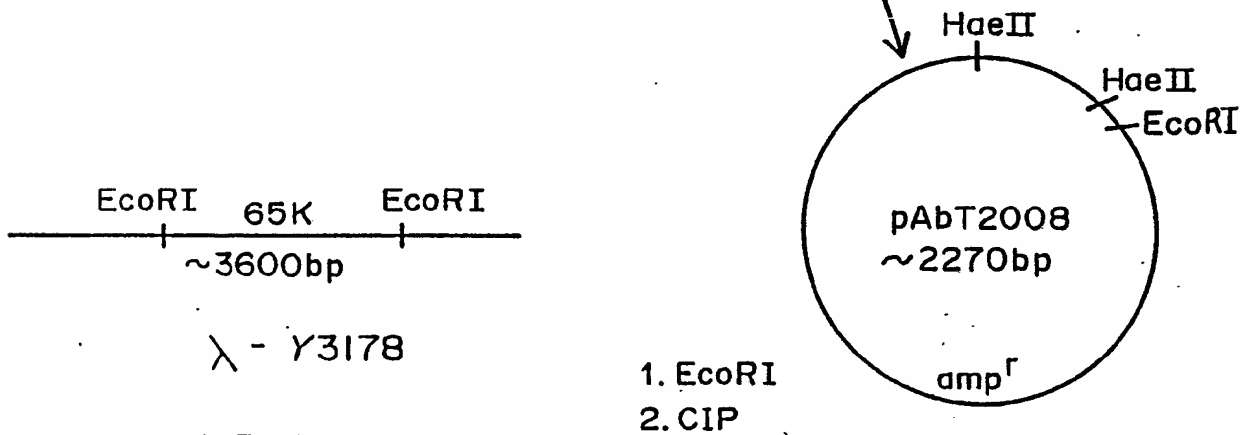


FIGURE 1b



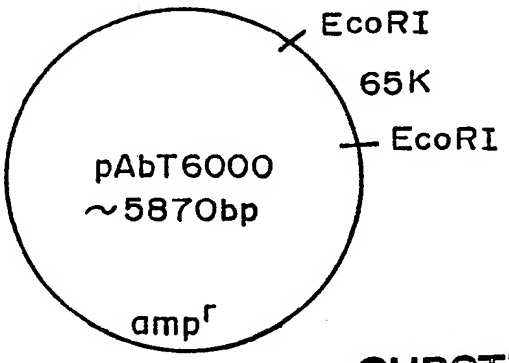
λ - Y3178

1. EcoRI

1. EcoRI
2. CIP

FIGURE 1c

Ligate



SUBSTITUTE SHEET

FIGURE 2a

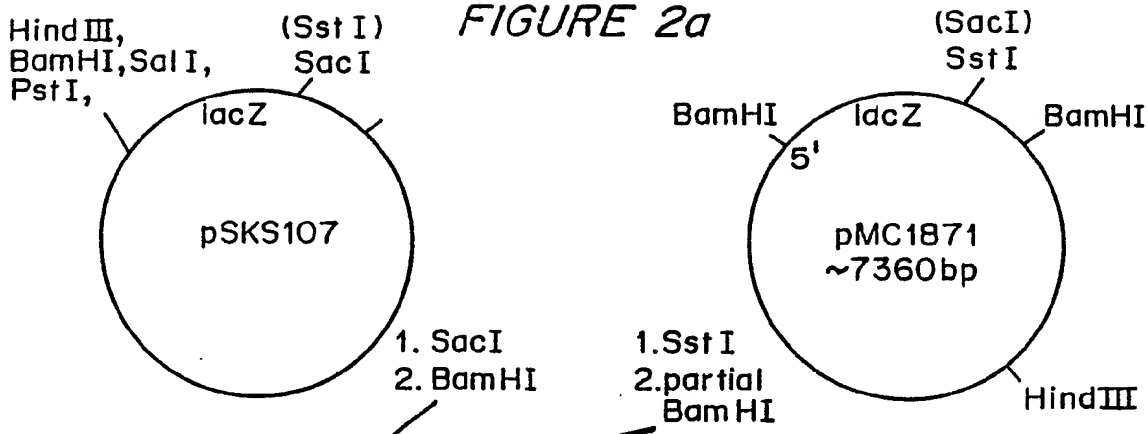


FIGURE 2b

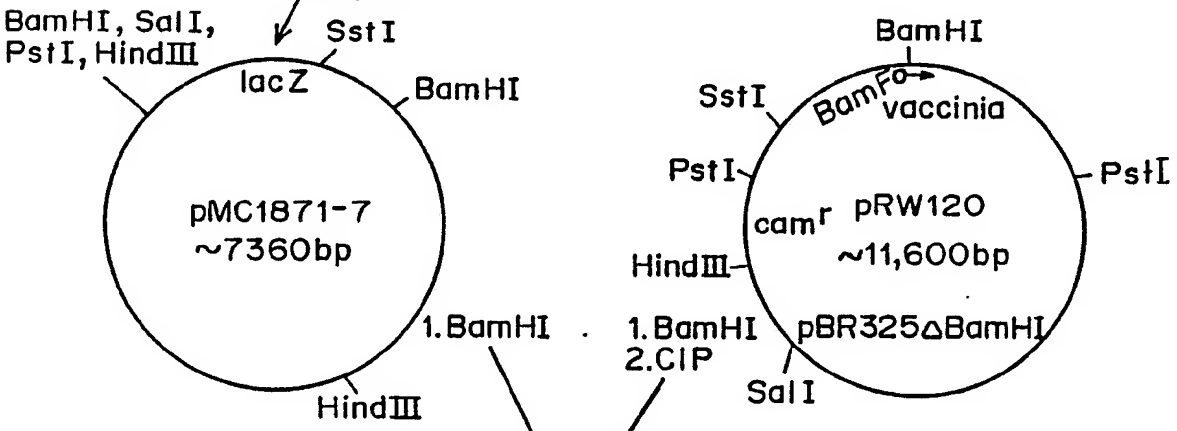


FIGURE 2c

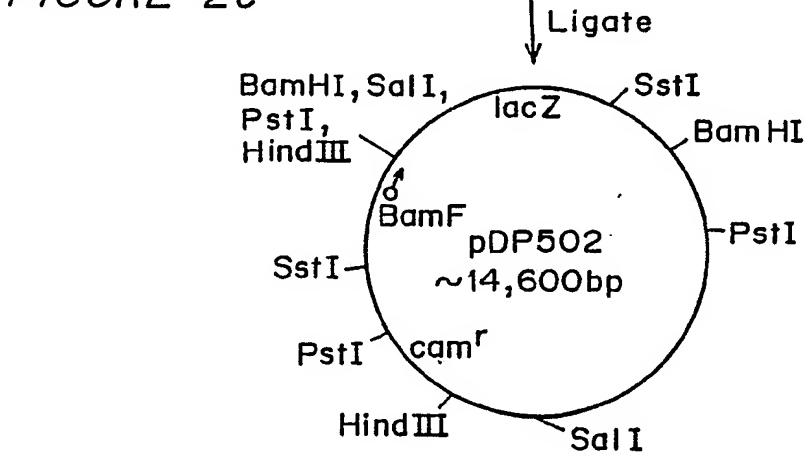


FIGURE 2d

Sequence at junction of vaccinia BamF and lacZ:

T AAT ATG ACG CTC GTC ATG GGA TCC GTC GAC CTG CAG CCA AGC TTG GCA

FIGURE 3A

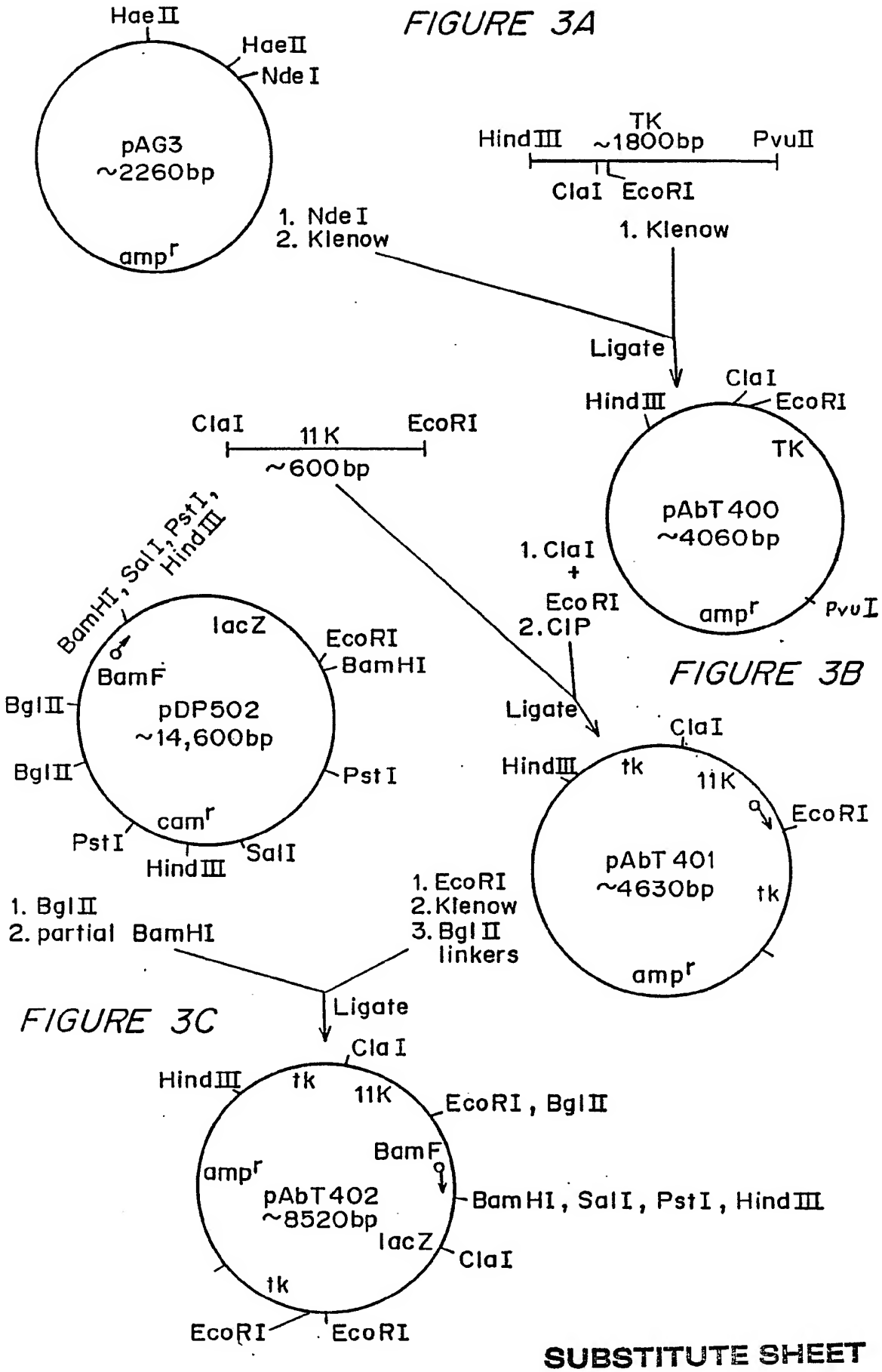
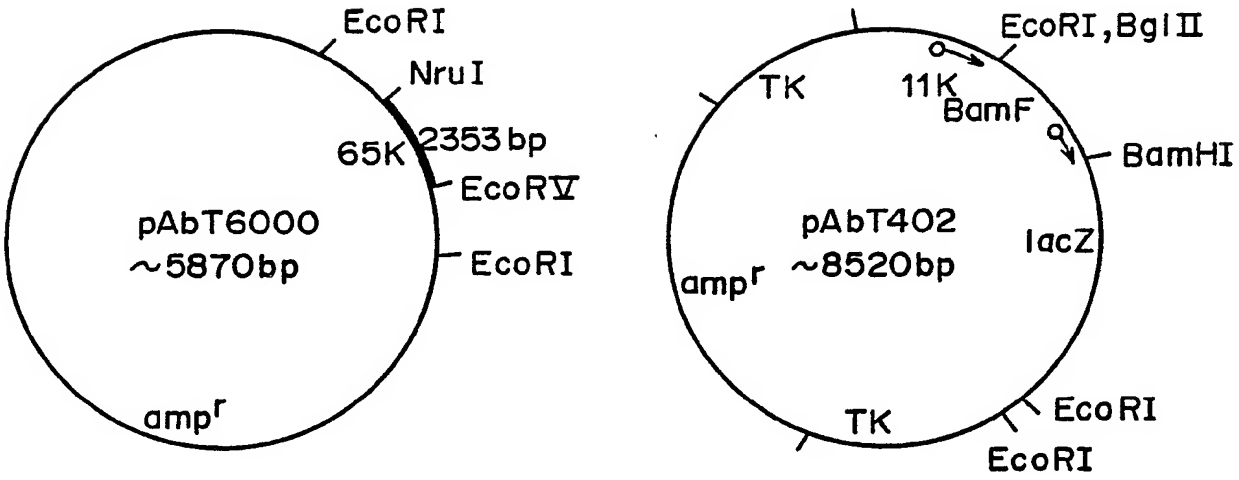


FIGURE 3B

FIGURE 3C

SUBSTITUTE SHEET

FIGURE 4a

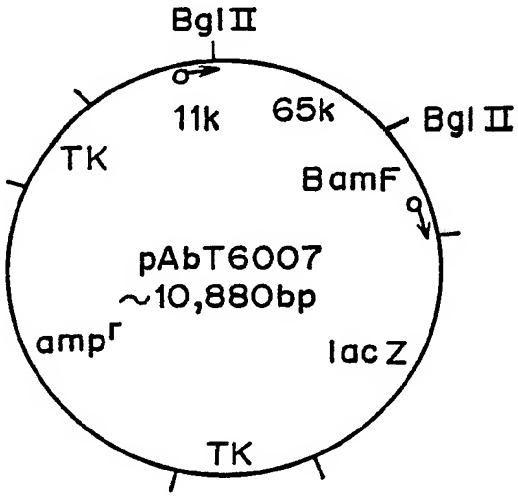


1. NruI + EcoRV
2. BglII linkers
   d(pGGAAGATCTTCC)

1. BglII
2. CIP

FIGURE 4b

Ligate



Fusion gene and protein generated by ligation of vaccinia 11K and M. leprae 65kd genes in pAbT6007: nucleotide sequence at junction of fusion gene and predicted amino acid sequence.

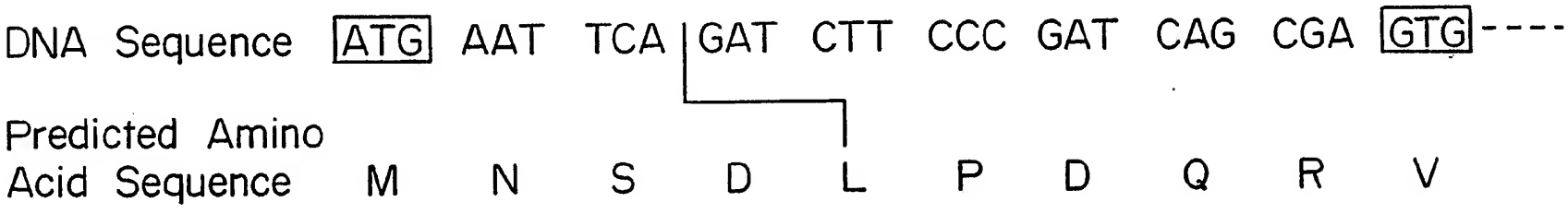


FIGURE 5

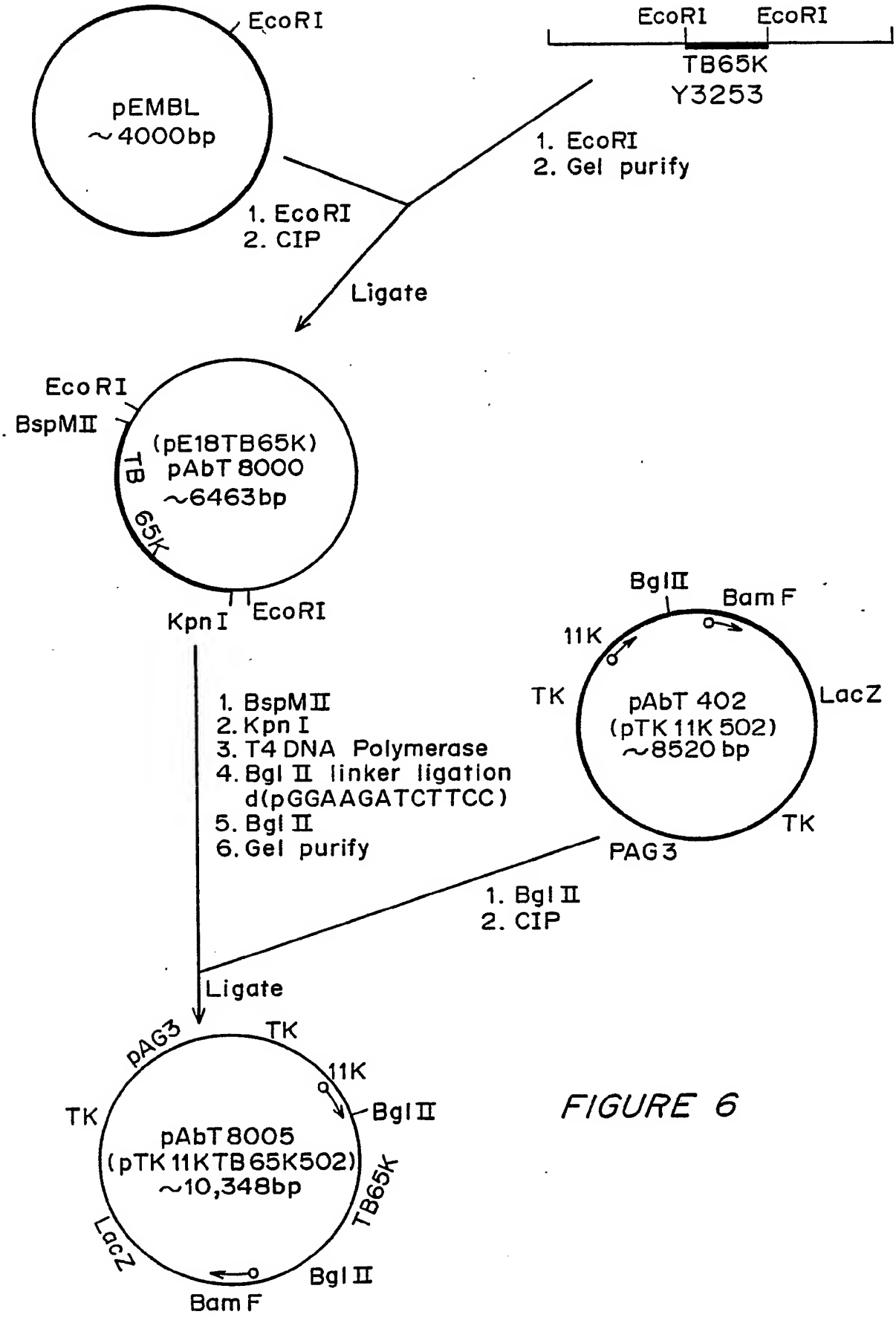

EcoRI
pEMBL
~4000bp
1. EcoRI
2. CIP
EcoRI EcoRI
TB65K
Y3253
1. EcoRI
2. Gel purify
Ligate
EcoRI
BspMII
(pE18TB65K)
pAbT 8000
~6463bp
TB
65K
KpnI
EcoRI
1. BspMII
2. Kpn I
3. T4 DNA Polymerase
4. Bgl II linker ligation
d(pGGAAGATCTTCC)
5. Bgl II
6. Gel purify
BglII
Bam F
11K
TK
pAbT 402
(pTK 11K 502)
~8520 bp
LacZ
TK
PAG3
1. Bgl II
2. CIP
Ligate
PAG3
TK
11K
BglII
TK
pAbT8005
(pTK 11K TB 65K 502)
~10,348bp
TB65K
LacZ
Bgl II
Bam F

FIGURE 6

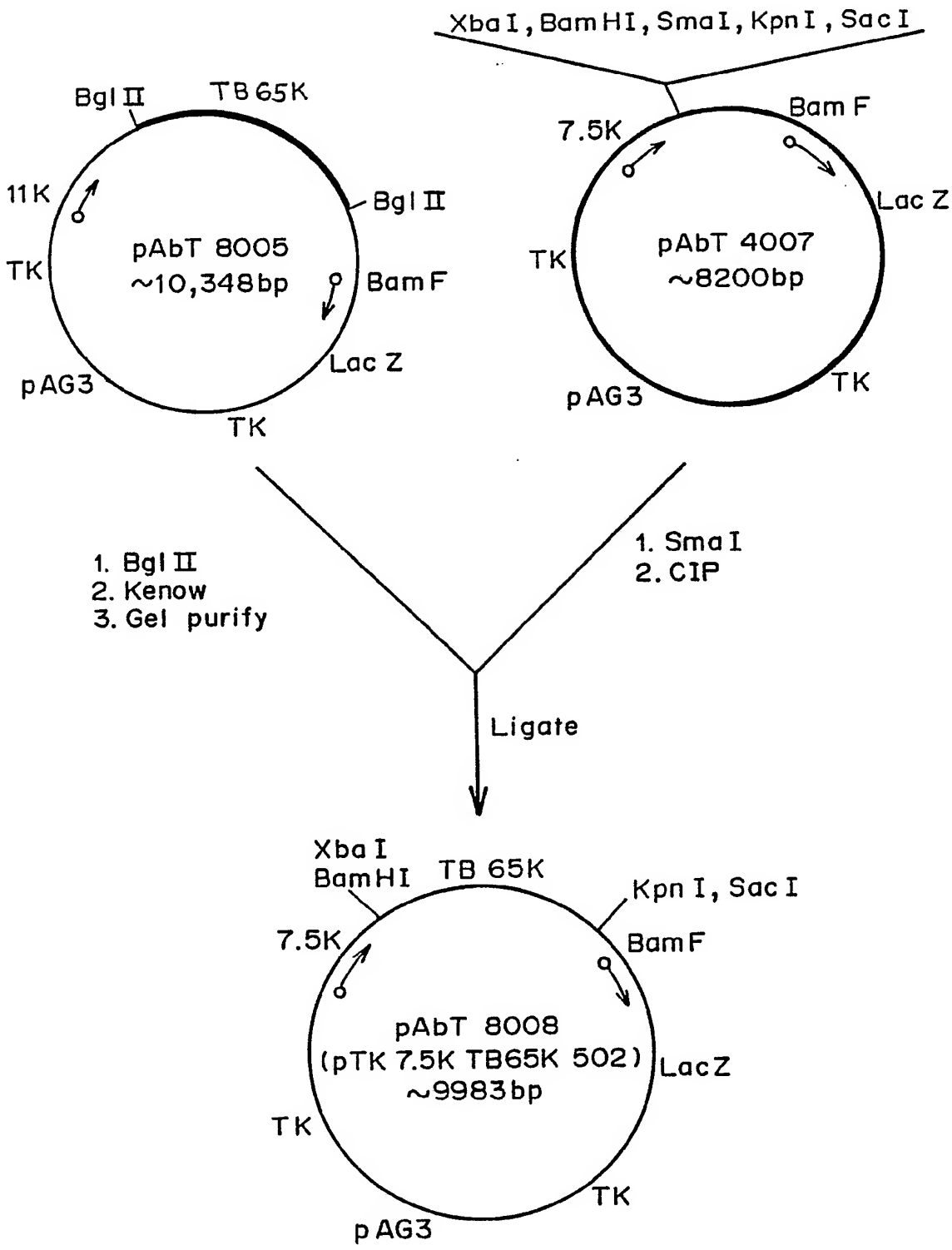

Xba I, Bam HI, Sma I, Kpn I, Sac I
Bgl II
TB 65K
11K
Bgl II
TK
pAbT 8005
~10,348bp
Bam F
Lac Z
pAG3
TK
7.5K
Bam F
Lac Z
TK
pAbT 4007
~8200bp
pAG3
TK
1. Bgl II
2. Kenow
3. Gel purify
1. Sma I
2. CIP
Ligate
Xba I
Bam HI
TB 65K
Kpn I, Sac I
7.5K
Bam F
pAbT 8008
(pTK 7.5K TB65K 502)
~9983bp
Lac Z
TK
TK
pAG3

FIGURE 7

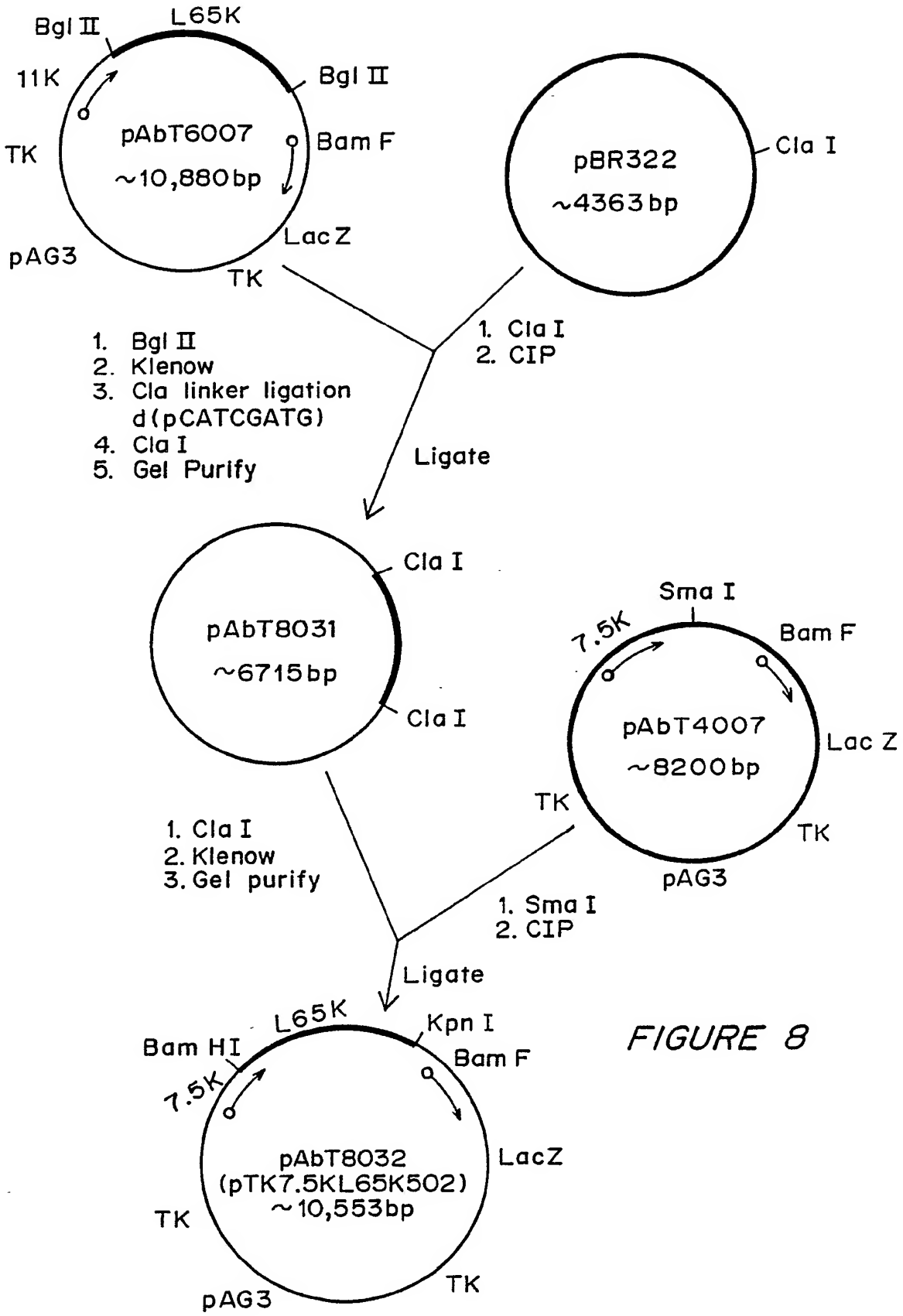
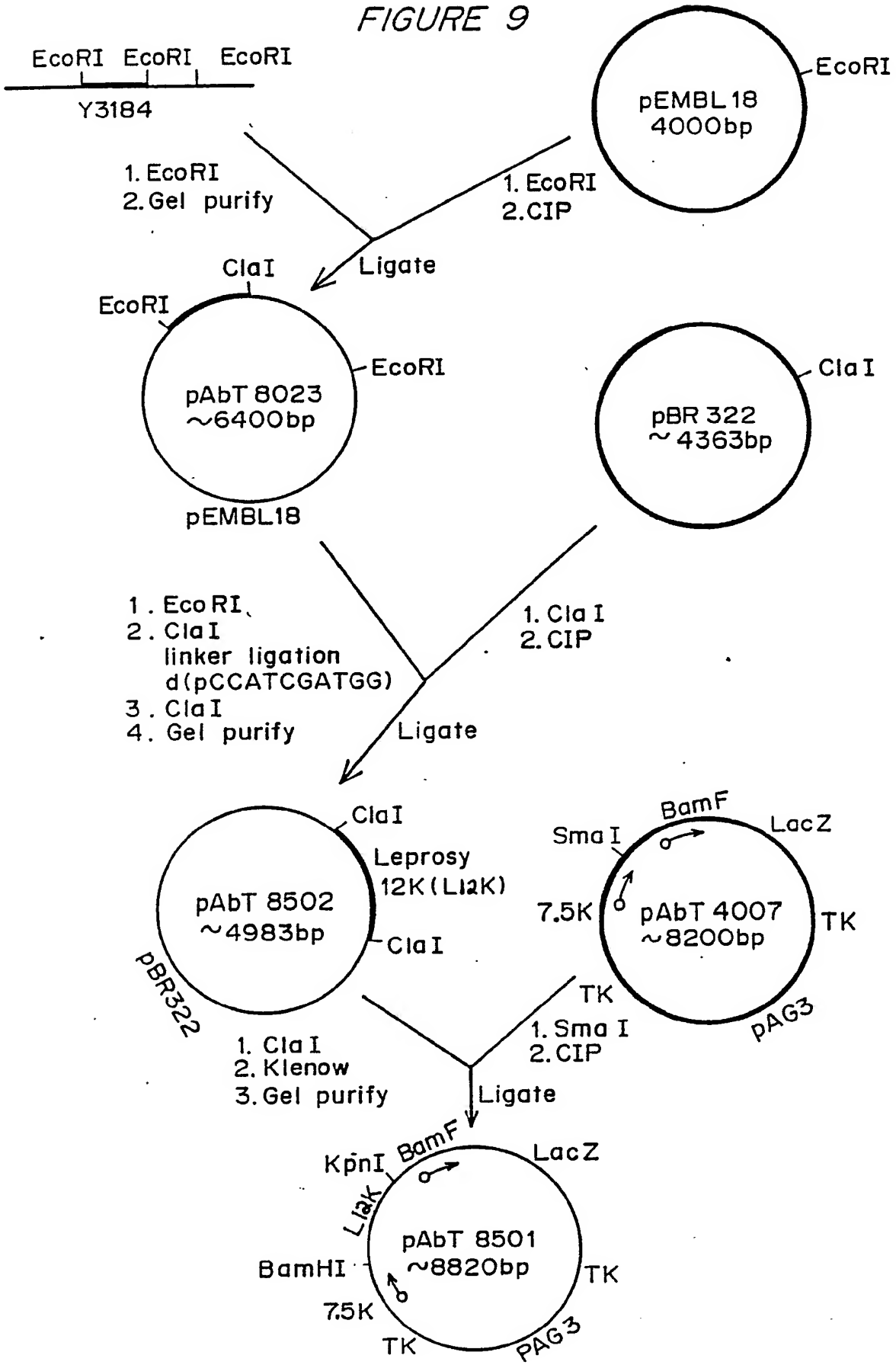


FIGURE 8

SUBSTITUTE SHEET

FIGURE 9


EcoRI EcoRI EcoRI
Y3184
1. EcoRI
2. Gel purify
pEMBL18
4000bp
EcoRI
1. EcoRI
2. CIP
Ligate
ClaI
EcoRI
pAbT 8023
~6400bp
EcoRI
pEMBL18
pBR 322
~4363bp
ClaI
1. EcoRI
2. ClaI
linker ligation
d(pCCATCGATGG)
3. ClaI
4. Gel purify
1. ClaI
2. CIP
Ligate
ClaI
Leprosy
12K (Li2K)
pAbT 8502
~4983bp
ClaI
pBR322
BamF
LacZ
SmaI
7.5K
pAbT 4007
~8200bp
TK
TK
pAG3
1. ClaI
2. Klenow
3. Gel purify
1. SmaI
2. CIP
Ligate
KpnI
BamF
LacZ
Li2K
pAbT 8501
~8820bp
BamHI
TK
7.5K
TK
PAG3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 87/02245

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 7/00; // A 61 K 39/04; G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proc. Natl. Acad. Sci. USA, volume 80, September 1983, (US), D. Panicali et al.: "Construction of live vaccines by using genetically engineered poxviruses: Biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin", pages 5364-5368 see the whole article, especially page 5367, last paragraph cited in the application	1-15
Y	EP, A, 0110385 (THE UNITED STATES OF AMERICA) 13 June 1984 see claims 10-13	1-15
Y	Nature, volume 316, 1 August 1985, R.A. Young et al.: "Genes for the major protein antigens of the leprosy parasite Mycobacterium leprae", pages 350-352 see the whole article cited in the application	1-5,9-15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th January 1988	- 3 FEB 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/US 87/02245

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proc. Natl. Acad. Sci, USA, volume 82, May 1985, (US), R.A. Young et al.: "Dissection of Mycobacterium tuberculosis antigens using recombinant DNA", pages 2583-2587 see the whole article --	1-3,6,11-14
Y	Infection and Immunity, volume 50, no. 3, December 1985, American Society for Microbiology, (US), J.E.R. Thole et al.: "Cloning of Mycobacterium bovis BCG DNA and expression of antigens in Escherichia coli", pages 800-806 see the whole article	1-3,11-14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8702245 SA 18735

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/01/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0110385	13-06-84	WO-A- 8402077	07-06-84
		AU-A- 2342484	18-06-84
		JP-T- 60500518	18-04-85